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DOCTORAL SCHOOL of
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The microbiological spectrum of invasive bacterial infections in Cambodian adults and implications for standard treatment guidelines.

Erika Vlieghe

Promotor:
Prof. Dr. Willy E. Peetermans

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Dissertation presented in partial fulfilment of the requirements for the degree of Doctor in Medical Sciences

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Biomedical Sciences Group
Faculty of Medicine
Department of Microbiology and Immunology

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'-Sincèrement, dites-moi votre pensée, avez-vous la certitude qu'il s'agit de la peste?

-Vous posez mal le problème. Ce n'est pas une question de vocabulaire, c'est une question de temps.

(…) Disons seulement que nous ne devons pas agir comme si la moitié de la ville ne risquait pas d'être tuée, car alors elle le serait'

Albert Camus, La Peste

Voor mijn ouders, die me naar het begin van dit alles geleid hebben

Voor mijn lieve mannen, die het van daar hebben overgenomen: Mark, Maarten en Wout

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List of abbreviations

BSI	bloodstream infection
CAP	community-acquired pneumonia
CI	confidence interval
CLSI	Clinical and Laboratory Standards Institute
CSO	clinically significant pathogen
DCS	decreased ciprofloxacin susceptibility
ESBL	extended spectrum beta-lactamase
EUCAST	European Committee on Antimicrobial Susceptibility Testing
HIV	human immunodeficiency virus
ITM	Institute of Tropical Medicine
LMIC	low and middle income countries
MDR	multi-drug resistance
MIC	minimal inhibitory concentration
MLST	multi locus sequence typing
MOF	multiple organ failure
MRSA	methicillin resistant *Staphylococcus aureus*
MSSA	methicillin susceptible *Staphylococcus aureus*
NGO	non-governmental organization
NTS	non-typhoid *Salmonella*
PCR	polymerase chain reaction
PFGE	pulsed field gel electrophoresis
PVL	Panton Valentin Leukocidin
RR	relative risk
SCC	Staphylococcal Chromosomal Cassette
SHCH	Sihanouk Hospital Centre of HOPE
SIRS	systemic inflammatory response syndrome
SMX-TMP	sulphamethoxazole-trimethoprim
SSTI	skin and soft tissue infections
ST	sequence type (MLST)
TSST	toxic shock syndrome toxin
WHO	World Health Organization
CCI	Charlson's Comorbidity Index
SAB	*Staphylococcus aureus* bacteremia

Dankwoord

‘The making of’ dit doctoraat speelde zich af gedurende de afgelopen zes jaar. Toch een zekere hap uit een mensenleven. Patiënten kwamen en gingen, ons Instituut doorstond een majeure hervorming, mijn man overleefde miraculeus een hartstilstand, onze zonen ontdekten de wereld, ons departement verloor een geliefd collega.

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Phnom Penh/Antwerpen/Meerbeek, najaar 2013

Chapter 1.
Introduction and rationale

Since the 1920's until present date, bacterial growth in blood cultures has remained the gold standard for the diagnosis of bloodstream infection, which may be considered the quintessential invasive bacterial infection.

1.1. Invasive bacterial infections

Bacteria are among the oldest living organisms on Earth [1]. While the majority of bacterial species are harmless to man and lead a saprophytic existence in the environment or are commensals of plants, animals and man, a small number of bacteria has been described which may cause human illness, the so-called pathogenic bacteria. These bacteria may colonize their hosts, cause local infection (*e.g.* skin or mucosal infections) or may invade the deeper tissues and internal organs and spread around the body through the bloodstream with or without secondary ('metastatic') infections at distance [2]. Invasive bacterial infections reflect the spread of pathogenic bacteria in otherwise sterile body sites and are mostly accompanied by an important inflammatory reaction of the hosts' body (*i.e.* sepsis), which may evolve into septic shock, multiple organ failure and even death [3]. Examples of these invasive bacterial infections include -amongst others- pneumonia, meningitis, peritonitis, pyelonephritis, deep organ abscesses, bone and joint infections, endocarditis and bloodstream infection. Severe bacterial infections may also complicate the course of major diseases at the global scale such as HIV/AIDS, tuberculosis and malaria [4, 5].

1.2. Bloodstream infection

Bloodstream infection (BSI) is defined as the presence of pathogenic bacteria in the blood from where they can spread to the rest of the body and may thus be considered the quintessential invasive bacterial infection. BSI can be primary (without clear infection focus) or secondary, spreading from an established infection focus in the body [6].

1.2.1. Diagnosis of bloodstream infections

Since the 1920's until present date, bacterial growth in blood cultures has remained the gold standard for the diagnosis of BSI [7]. Blood cultures are, in contrast to cultures from non-sterile sites (such as respiratory or urinary tract samples) relatively easy to perform, standardize and interpret. However, blood cultures require the presence of a microbiological laboratory and well-trained staff, and have a minimal turnaround time of 1-2 days before first results are known, and another 48-72 h to obtain final identification and susceptibility test results (Figure 1) [8]. Recently, several rapid diagnostic tests have been developed to improve speed of diagnosis either on grown blood cultures or directly on the sample, including (but not limited to) polymerase chain reactions (PCR), micro-arrays and matrix-assisted laser desorption ionization time-of-flight mass spectrometry

(MALDITOF)[8]. However promising, most of these techniques require also expensive hardware, consumables and skilled technicians, which has precluded their worldwide availability so far.

The sad reality is that microbiology laboratories in many low and middle income countries (LMIC) - where existing- often lack basic funding, skilled staff and a stable reagents' stock [9, 10]. More priority is often given to tests funded through vertical programs (such as for tuberculosis, malaria, HIV) or to particular analyses with more commercial potential (*e.g.* within the context of infertility diagnosis). In the past decade more attention has been asked by international researchers for the public health potential of microbiology laboratories across the world [11, 12].

1.2.2. Epidemiology of bloodstream infections worldwide

BSI may occur as community-acquired infections, or can be associated with recent (health care associated) or ongoing (nosocomial) contact with the health care setting. Population-based studies on the incidence of BSI are scarce. Data from high-income settings in the US and Europe indicate that BSI occur in about 140-160 per 100.000 population per year [13], and that the incidence has increased by a factor 4 or more in most study settings over the past 30 years. The yearly number of patients with BSI has been estimated at 575.000-677.000 in North America, and 1.200.000 in Europe[14]. For LMIC, data on BSI are largely lacking due to the extreme scarcity of well-functioning microbiology laboratories in sub-Saharan Africa, and in rural or remote areas in Asia and South-America [12]. However, the decline of malaria incidence, the large patient population with the human immune deficiency virus (HIV) and the worldwide emergence of antibiotic resistance have led to more attention and scientific interest over the past decade for BSI and other invasive bacterial infections in LMIC. A population-based study from Kenya estimated the annual incidence of pediatric BSI at 2.440 cases per 100.000 children younger than 2 years, and at 1.192 cases per 100.000 children younger than 5 years of age [15]. In smaller-scale studies from LMIC, BSI have been repeatedly found major causes of morbidity and mortality in vulnerable patient groups such as neonates [16], under-fives [17] and immune depressed adults such as HIV-patients [4, 18]

1.2.3. Spectrum of pathogens causing bloodstream infection

The spectrum of bacteria causing bloodstream infection is influenced by several factors, including the geographical setting, patients' age range, vaccination status, co-morbidity (such as HIV-infection, malnutrition, diabetes mellitus or cancer) and the infection setting (community-acquired, health care associated or nosocomial).

In temperate, high income settings such as Europe and the US, BSI is increasingly caused by Gram-positive pathogens such as *Staphylococcus aureus*, coagulase negative staphylococci and

Streptococcus pneumoniae, besides *Escherichia coli* as the main Gram-negative pathogen [13, 19]. This pattern probably reflects in part the growing population in these regions of older and/or immune depressed patients who frequently require medical procedures and devices, and where the borders between community-acquired and healthcare-associated infection become more and more blurred.

In other parts of the world, other, mostly Gram-negative pathogens play a prominent role as causes of BSI. Non-typhoid *Salmonella* spp. (NTS) are among the most frequent pathogens of BSI in sub-Saharan Africa [20]; *Salmonella* Typhi in South and Southeast Asia [21], *Burkholderia pseudomallei* in Northern Australia and Northeast Thailand [22, 23], *Klebsiella pneumoniae* in sub-Saharan Africa and East Asia [24]. *Burkholderia pseudomallei* is a non-fermentative Gram-negative bacterium prevalent in surface waters and soil in defined areas of (mainly) Southeast Asia, Northern Australia and is the etiological agent of melioidosis. Human infection can occur through inhalation, ingestion or skin contact with infected water or mud. The disease presents as a purulent, abscess-forming infection of skin, lungs, bones and deep organs, with a clinical course ranging from mild skin disease to life-threatening septic shock [25]. *Klebsiella pneumoniae*, in high-income temperate regions known as a cause of nosocomial infections, is an established cause of community-acquired bacteremic pneumonia in alcoholics in southern Africa and Taiwan [24] and is the cause of a specific type of community-acquired liver abscesses in Taiwan and other parts of East Asia *i.e.* '*Klebsiella pneumoniae* liver abscess'. Also in (South) East Asia, the Gram-positive coccus *Streptococcus suis* acts equally as regionally relevant pathogen. This zoonotic pathogen colonizes the nasopharyngeal tract in pigs and may cause invasive disease in piglets and pigs. Humans may acquire the bacterium after contact with infected animals or their products or through ingestion of undercooked meat; infection is often severe and presents commonly as meningitis and/or BSI. The majority of human *S. suis* infections worldwide occur in Southeast and East Asia, and the pathogen has been described as the most common cause of bacterial meningitis in Vietnam [26].

The reasons for these geographical differences may be explained by local rates of poverty, crowding and lack of hygiene (*e.g. Salmonella enterica*), close contact between humans and animals (*e.g.* NTS, *S. suis*) high prevalence of HIV-seropositivity and malnutrition in children (NTS) [27, 28], presence of the typical environment, climate or season required (*B. pseudomallei, K. pneumoniae*) [29]. Yet, for many places in the world these local differences in bacterial spectrum are neither well described nor explained.

This lack of knowledge on regionally relevant pathogens may cause mismatches in antibiotic choices for patients presenting with severe infections, which may eventually impact patient outcomes [30, 31], especially in this era of quickly emerging antibiotic resistance worldwide.

1.2.4. Outcome of patients with bloodstream infection

As displayed in Table 1, the clinical outcome of a BSI is the resultant of several pathogen characteristics (*i.e.* virulence, quantity of pathogens, antibiotic resistance,…) and host factors (co-morbidity, age, gender, inflammatory response,…)[32]. BSI has been associated with important morbidity at short and longer term [33] and with considerable mortality. In high-income settings in Europe and the US, case fatality of BSI ranges between 13 and 30% [14]. With an estimated total yearly death toll of 79.000-94.000 in North America and 157.000 in Europe, Goto and colleagues found BSI to rank among the top seven causes of death in these regions, which is higher than for any other infection, including influenza and pneumonia combined [14]. However, obtaining accurate, population-based data proved to be a very difficult task in high-income settings, let alone in regions with a weaker healthcare- and administrative infrastructure. In addition, the diagnosis 'BSI' is often overlooked in mortality statistics, as the more visible and easier to diagnose source infections (*e.g.* pneumonia or urinary tract infection) are usually coded as the final diagnosis.

Besides adequate sepsis care, the administration of appropriate antibiotic therapy is the cornerstone of successful management of the patient with BSI [34, 35]. This includes the selection of the most appropriate drug in a correct dosing scheme and for an effective duration, all based ideally on evidence and accurate knowledge of local bacterial pathogens and their resistance patterns. However, the worldwide emergence of antibiotic resistance has led to drastic changes in the choice of antibiotics for empirical (*i.e.* before or without the availability of bacterial cultures) or directed treatment (*i.e.* adapted to bacterial cultures) in national or international treatment guidelines, with an evolution towards an increased use of broad spectrum antibiotics [36].

1.3. Antibiotic resistance

Antimicrobial resistance has been defined by the WHO as 'resistance of a microorganism to an antimicrobial medicine to which it was previously sensitive' [37]. It can be detected 'phenotypically' by determining the minimal inhibitory concentration (MIC) of an antibiotic required to kill a defined amount of bacteria, either through broth dilution methods or pre-defined teststrips on agar plates (*i.e.* Etest) (Figure 2). An alternative and cheaper technique is to observe the growth of bacteria on an agar plate around disks containing a well-defined concentration of an antibiotic (disk diffusion);

the diameters of growth inhibition generated by the antibiotic disk correlate also with breakpoints [38, 39]. Breakpoints are those levels of MIC above which the antibiotic is considered not suitable anymore for treating an infection with this bacterium in a patient; these are based on a combination of findings from pharmacokinetics, pharmacodynamics, toxicity- and clinical outcome studies in combination with knowledge on the available antibiotic formulations, infection site and bacterial species [38, 40]. Over the past decades, genetic testing methods (such as PCR and sequencing) have been used increasingly to screen for certain genes conferring resistance [41].

1.3.1. A brief history of antibiotic resistance

Antibiotic resistance is probably as old as the existence of bacteria themselves [42], but the increasing use of antibiotics in humans and animals in the past seven decades has precipitated the selection of those bacteria resistant to the most commonly used antibiotics of that era. As shown in Figure 3, at each introduction of a certain antibiotic class, bacterial resistance occurred within the following years [43]. Penicillin and oxacillin resistance in *S. aureus* have been for a long time the most prominent resistance problems since the 1950's until the advent of highly technological, contemporary medicine in the 1970's which heralded an exponential increase and variety of antibiotic development, use and emergence of resistance mechanisms [43]. Until the 1980's, antibiotic resistance was mainly found in nosocomial or healthcare associated settings, in high-income settings of Europe and North America. This is illustrated by the evolution of methicillin-resistant *S. aureus* (MRSA), which was considered a mere nosocomial infection until the emergence of community-acquired MRSA in the early years of the 21st century [44, 45]. Whereas MRSA rates appear to be declining nowadays in adults from high income settings in Europe and the US [46], the opposite holds true for Gram-negative infections [47, 48].

In the 1980's, the first extended spectrum beta-lactamases (ESBL) were described in Gram-negative bacterial infections within a nosocomial context; this was followed by a steep increase of ESBL occurrences across the world, and increasingly in community-acquired infections [49]. Nowadays, Asia is the unfortunate 'market leader' in prevalence, disease burden and genetic diversity of ESBL, with CTX-M being the most successful ESBL-type worldwide [43, 49]. For instance, ESBL rates among community-acquired *E. coli* isolates from clinical samples are higher than 60% in China, and more than 80% in India [50, 51]. In addition, pathogens carrying ESBL display frequently, besides the expected resistance for most penicillins and cephalosporins, co-resistance to other antibiotic classes which makes these pathogens multidrug resistant. This phenomenon has led to an increased use of carbapenem antibiotics over the past decades, which, in turn led to the collateral damage of selection for carbapenem resistance in an increasing variety of Gram-negative pathogens [50]. Initially

a problem of nosocomial infections with non-fermentative Gram-negative rods (*e.g. Pseudomonas aeruginosa*, *Acinetobacter* spp.), carbapenemases have started to occur in *Enterobacteriaceae* from community-acquired infections [52]. In 2008, the New Delhi metallo-betalactamase-1 (NDM-1), was described in patients living in or following recent hospitalization in India and has spread from there to other Asian countries and worldwide along international travel [53, 54]. Its presence in the environment and ability to accumulate various other resistance genes are of particular concern [55, 56]. In the past decade, a wide range of genes conferring carbapenem resistance in a variety of Gram-negative pathogens has spread across the Asia and in other parts of the world [57].

Salmonella Typhi and non-typhoid *Salmonella* spp., both common pathogens in LMIC, underwent a different resistance evolution. Multidrug resistance (*i.e.* co-resistance for the amoxicillin, sulphamethoxazole-trimethoprim (SMX-TMP) and chloramphenicol) emerged in the late 1980's in southern Vietnam [58]. Soon afterwards fluoroquinolones entered the market, followed by the first point mutations at the genes encoding *gyr*A or *par*C, leading to decreased ciprofloxacin susceptibility, which has now widely spread among 50-90% of *Salmonella* Typhi isolates in South and Southeast Asia [57, 58]. The zoonotic non-typhoid *Salmonella* serovars have acquired more frequently other resistance genes, including ESBL and very rarely carbapenemases [59, 60].

Antibiotic resistance has now affected nearly every clinically relevant bacterial species across the world, and the evolution followed a clear trend spreading from the healthcare setting into the communities, with an actual emphasis on plasmid-related resistance problems in Gram-negative pathogens.

1.3.2. Mechanisms of bacterial resistance

A wide variety of genetic mechanisms may be underlying phenotypic antibiotic resistance; this depends on the bacterial species, circulating clones and the type of antibiotic and is in permanent evolution. As shown in Figure 4, bacteria may become resistant to a certain antibiotic by blocking its entrance or actively pumping it out, by changing the structure of key proteins where antibiotics attach or by altering or degrading the antibiotic molecules [61]. This resistance capacity may be caused by mutations of the original bacterial genetic material (*i.e.* 'chromosomal mechanisms'), which can be transmitted only 'vertically' to the next generation of bacteria. This is the case for instance in *Enterobacteriaceae* displaying resistance for fluoroquinolones through point mutations in genes encoding *gyr*A or *par*C of the Quinolone Resistance Determining Region [62]. Alternatively, resistance may also occur through the acquisition of mobile elements containing resistance genes, such as plasmids. These can however be transmitted vertically and horizontally, across bacterial species,

which allows a much faster spread. Many recently emerging resistance mechanisms are of this second type, including ESBL and several carbapenemases including NDM-1 [63].

1.3.3. Causes of antibiotic resistance

The causes of this worldwide resistance 'epidemic' are multiple and complex, and are to be found at each level of the antibiotic use chain, including the prescribers, dispensers, patients and the health care facilities; they are to a certain extent symptomatic for broader economic and regulatory problems present in many LMIC.

Because of poverty, crowding, malnutrition, limited provision of safe water and sanitation and variable vaccine coverage rates, the burden of infectious (febrile) diseases is high in many LMIC, especially at the extremes of life [64-66]. Lack of education in the general public and easy access to over-the-counter drugs has led to the common idea of antibiotics as a uniform solution to nearly every physical ailment [67]. Many LMIC lack (enforced) regulations regarding drug dispensing so that antibiotics are commonly bought without prescription at pharmacies and small shops. Knowledge of local dispensers and drug sellers is usually poor, and substandard or counterfeit drugs are common at local markets [68, 69]. Irrational antibiotic prescriptions occur when local physicians work with limited medical training, outdated guidelines and scarce diagnostic support and may be influenced by financial incentives to upgrade their salaries [70, 71]. Finally, health care settings in LMIC often lack essential infection control measures, allowing spread of pathogens among patients and between the hospital and community setting.

Probably even more important than antibiotic use in human medicine is its use for therapeutic or growth promoting purposes in the agricultural sector at large, including for livestock, aquaculture and even horticulture [72, 73]. In a world with a global economy and millions of people, goods and animals traveling between countries, new resistance profiles can spread very fast [74] (Figure 5).

1.3.4. Global impact of antibiotic resistance

Antibiotic resistance has become a global problem which has increasingly affected all continents for the past decades. Each time a new drug class has been introduced on the market, new resistance mechanisms may be selected out, allowing the resistance problem to expand to these new drugs as well. In a recent report, the US Centers for Disease Control and Prevention estimate that antibiotic resistance affects yearly more than 2 million US citizens, leading to death in 23.000 persons [75]. Likewise, MRSA, cephalosporin resistance in *Enterobacteriaceae* and carbapenem resistance in *Pseudomonas aeruginosa* have been estimated to cause 5.400, 8.000 and 10.200 extra deaths in Europe each year. The majority of those infections is associated with BSI [76].

Even though its scale is not yet well assessed in LMIC, there is evidence that bacterial resistance is quickly becoming a very important health problem for most resource limited countries as well. They are often hardest hit by this new epidemic (*e.g.* multidrug resistant *Salmonella* Typhi and 'superbugs' such as NDM-1 positive *Escherichia coli*) [36]. The problem of antibiotic resistance and subsequent inadequate antibiotic therapy is especially relevant in invasive infections: several of these infections (*e.g.* BSI, pneumonia and meningitis) are intrinsically associated with a higher morbidity and mortality, on top of which accumulates the additional burden of morbidity and mortality brought along by antibiotic resistance through delayed or inappropriate patient management.

Given its impact on morbidity, mortality and the use of more expensive broad spectrum antibiotics, antibiotic resistance is an important cause of increased health care expenditures across the world [36]. Again, patients and societies in LMIC are hardest hit, with costs caused by antibiotic resistance burdening the already tight healthcare budgets [77].

In a recent call for global efforts to tackle antibiotic resistance, the World Health Organization (WHO) has listed a number of action points including strengthened surveillance of bacterial resistance and of antibiotic use and the implementation of antibiotic stewardship and infection control policies [76].

1.3.5. Surveillance of antibiotic resistance

Surveillance of a certain disease describes its distribution in the population, and follows its changes and trends over time [78]. Surveillance may be community- or hospital based, mono- or multicentric, coordinated at local, regional, national or international level, and performed episodically or continuously. For the surveillance of antimicrobial resistance, microbiological diagnosis is indispensable, ideally combined with clinical data including information on morbidity and mortality. Given its central role in the selection of antibiotic resistance, antibiotic use should also be surveyed to complement microbiological surveillance data. Surveillance data are needed to define or update standard treatment guidelines, and they may guide appropriate drug supplies, identify the need for implementation of infection control measures and suggest or monitor new interventions.

According to recommendations of the WHO [78], surveillance should focus on diseases with greatest public health importance and/or readily transmissible infections. The high clinical relevance of BSI and its association with a wide range of bacteria, unequivocal diagnosis and ubiquitous prevalence across different ethnic groups and geographic locations make them a good model for resistance surveillance in invasive bacterial infections at large.

For feasibility reasons, most surveillance programs use data obtained as part of routine patient care, and focus in their surveillance activities on key pathogens which include mainly *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella* Typhi, *Shigella* spp., *Neisseria meningitidis*, *Neisseria gonorrhoeae* and *Pseudomonas aeruginosa.*

For any type of surveillance, quality assurance is a prerequisite, in its pre-analytical as well as in the analytical and post-analytical phases. This implies correct sampling, transport and registration of samples, standardized testing methods, the use of validated interpretation rules (such as those of the Clinical and Laboratory Standards Institute (CLSI) or European Committee on Antimicrobial Susceptibility Testing (EUCAST) and timely communication of results to prescribers and policy makers. However, in LMIC, these strict requirements are often not met due the absence or dire state of the local microbiology laboratories as mentioned above. Therefore, surveillance activities are often biased towards richer and/or accessible (urban) populations, to 'fashionable' or epidemic pathogens (*e.g.* influenza and other emerging respiratory viruses) or to diseases for which 'vertical' programs exist, such HIV, malaria, tuberculosis (TB). For instance, large multicentric commercial surveillance studies (*e.g.* SENTRY, ANSORP, SMART) addressed historically only the high income settings in Asia, the Western Pacific and South Africa although over the past years middle income countries (such as Indonesia, Thailand, Vietnam and the Philippines) and the emerging economies (India, China) have been gradually included in these projects because of increasing laboratory capacity of sufficient quality. However, for the poorest countries in rural Asia (*e.g.* Cambodia, Laos, Myanmar, Bangladesh,…), microbiological services within the country and thus sound data on invasive bacterial illnesses remain scarce.

1.4. The study setting: Cambodia

1.4.1. The country

Cambodia is a Southeast Asian country situated between Thailand, Vietnam and Laos, with an estimated population of about 15 million inhabitants (Figure 6, 7). The country, with a Gross National Income per capita of 820 US $ and still recovering from decades of civil war, is considered a low-income country [79]. Eighty percent of the population lives in rural areas, 30% lives of less than 0.59 US $ per day. Compared to other countries in the region, the burden of communicable diseases is disproportionally high, including severe bacterial infections [80] which is a challenge in combination with the scarcity of functional microbiology laboratories in the country and a public health sector

under reconstruction (Figure 8, 9). Several healthcare systems co-exist, including public, private for profit, non-governmental and traditional healers. The private medical market is largely unregulated. Health care insurance is -to a certain extent- available for the 25% poorest through so called 'health equity funds' [81]. This implies that the majority of health expenditures are paid out of pocket. For serious health problems, poor people often end up borrowing money at inflated interest rates, which not rarely leads to household economics' catastrophes [82, 83]. Within this context, buying drugs at one of the numerous pharmacies is often the most affordable action in case of illness [84].

Similar to other low-resources Asian settings, inappropriate prescribing and dispensing of antibiotics is probably very common. While solid data for antibiotic usage in Cambodia are absent so far, there is a large body of published information on poor quality anti-parasitic drugs (in particular antimalarial drugs) in the country and in the larger Southeast Asian region [85]. National standard treatment guidelines for bacterial diseases date back to 1999, an actual revision process is ongoing. The use of antibiotics in agriculture is largely unknown but estimated by local experts to be high and largely unregulated [86, 87].

1.4.2. Collaboration with Sihanouk Hospital Centre of HOPE

Sihanouk Hospital Centre of HOPE (SHCH) is a Non-Governmental Organization (NGO) 40-bed hospital for adults in Phnom Penh, Cambodia. It provides clinical care for the local and referred citizens with specific focus on patients with the human immunodeficiency virus (HIV) and the chronically ill. During the study period 2007-2010, the hospital and its associated clinics provided about 61,000 outpatient visits and 1200 admissions per year of patients from across Cambodia. After the opening of new clinics attached to the hospital, these figures evoluated to 135,000 outpatient visits and about 1,000 hospital admissions per year (2013 data).

In the year 2000, SHCH and the Institute of Tropical Medicine, Antwerp (ITM) started an institutional collaboration focusing on capacity building in the diagnosis and management of infectious diseases. In this period in Cambodia, antiretroviral treatment was virtually absent, and HIV/AIDS-related opportunistic infections were common presentations among SHCH's patients. The delivery of evidence based care of opportunistic infections and HIV-infection was the priority of this framework agreement (FA2) with the Belgian Directorate General of Development Cooperation (DGD). The expansion of the laboratory and the consecutive presence of expatriate infectious diseases' specialists led to an interest for infectious diseases at large (including bacterial infections) and the opening of a dedicated microbiology laboratory in 2005 supported by the framework agreement. While the roll-out of antiretroviral therapy had become a success thanks to several rounds of the Global Fund [88], increasing time and budget was spent on bacterial surveillance and later antibiotic

stewardship activities. In 2007 a dedicated project on antibiotic resistance was launched, focusing on building microbiology laboratory capacity, surveillance of bacterial resistance and translation of its findings into clinical practice. In July 2007 a prospective blood culture study was initiated in patients presenting with presumed BSI. This study was the backbone of this PhD thesis, which describes the combined microbiological and clinical findings of BSI and its key pathogens among the adult patient population attending SHCH.

1.5. Main objectives of the study

I. To define the key pathogens causing invasive bacterial infections in Cambodian adults and to describe the patterns and mechanisms of antibiotic resistance in these pathogens **(Chapter 2)**

II. To provide detailed epidemiologic description of five local key pathogens of BSI in Cambodian adults with particular clinical presentation and microbiological characteristics: *Burkholderia pseudomallei* **(Chapter 3)**, *Salmonella* spp. **(Chapter 4)**, *Escherichia coli* **(Chapter 5)** *Staphylococcus aureus* **(Chapter 6)**, and *Streptococcus suis* **(Chapter 7)**.

III. To provide supporting evidence for locally adapted standard treatment guidelines for presumed BSI in Cambodian adults **(Chapters 2-8).**

Figure 1. Diagnosing bloodstream infections in the Democratic Republic of the Congo (Photograph by Jan Jacobs)

Table 1. Factors associated with adverse outcome of bloodstream infections*.

Pathogen factors
polymicrobial infection
presence of virulence factors (intrinsic or acquired)
high inoculum
resistance to the most effective or available antibiotic(s)
Host factors
male gender
extremes of age
immune depression (congenital or acquired)
co-morbidity (*e.g.* chronic organ failure, diabetes mellitus, obesity)
inflammatory response intensity (innate and acquired)
Infection episode factors
infection severity (*e.g.* association with septic shock, multiple organ failure,…)
difficult-to-treat underlying affected organ (*e.g.* lung, CNS, peritoneum)
unknown or non-drainable infection focus
delayed essential sepsis care (*i.e.* fluid resuscitation, oxygenation)
delayed appropriate empiric and directed treatment

** adapted from: Weinstein Clin Infect Dis 1997; Hoursom Postgrad Med J 2011; Heffner Clin Infect Dis 2010; Angus New England J Med 2013*

Figure 2. Assessment of antibiotic resistance by Etest left) and disk diffusion (right)

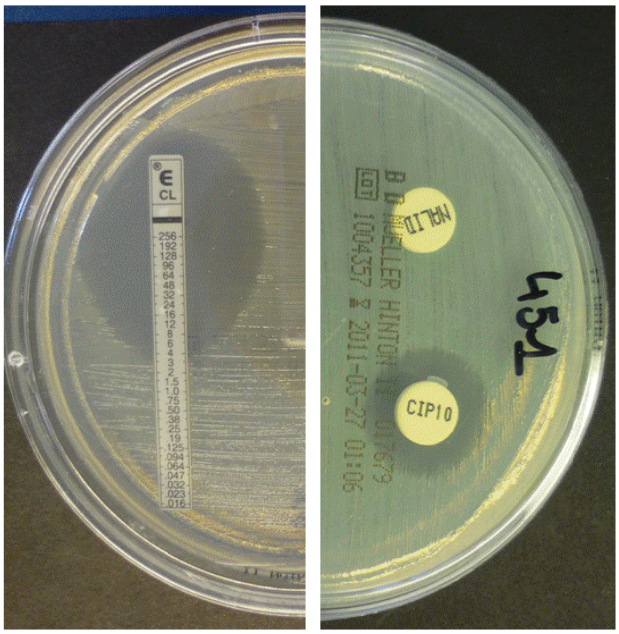

Figure 3. Evolution of antibiotic use and resistance over the past 80 years. (From: Molton J.S. et al, Clinical Infectious Diseases 2013;56(9):1310–8.)

AmpC: AmpC–producing *Enterobacteriaceae*; ESBL: extended-spectrum beta-lactamase–producing *Enterobacteriaceae*; KPC: *Klebsiella pneumoniae* carbapenemase–producing *Enterobacteriaceae*; MRSA, methicillin-resistant *Staphylococcus aureus*; NDM-1, New Delhi metallo-beta-lactamase-1–producing *Enterobacteriaceae*; PRSA, penicillin-resistant *Staphylococcus aureus*; VRE, vancomycin-resistant Enterococcus; VRSA, vancomycin-resistant *Staphylococcus aureus*.

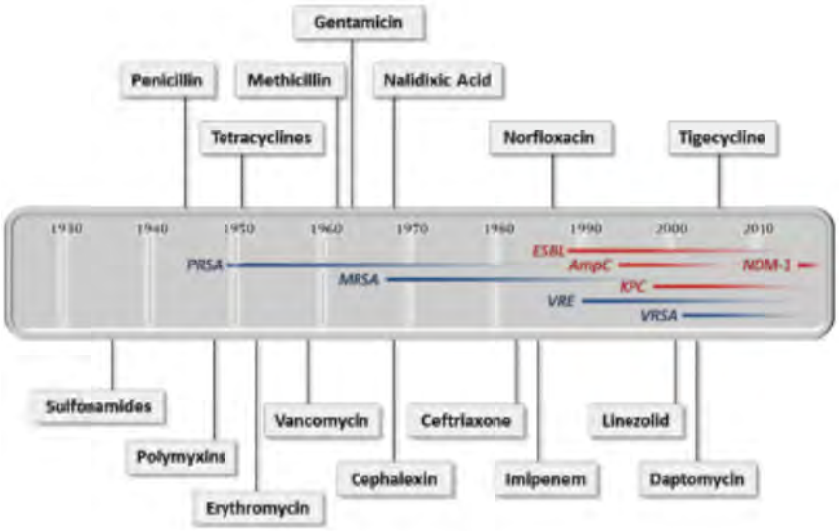

Figure 4. Diversity of bacterial resistance mechanisms. (From: Stuart L and Marshall B. Nature Medicine 2004 Dec;10(12 Suppl):S122-9)

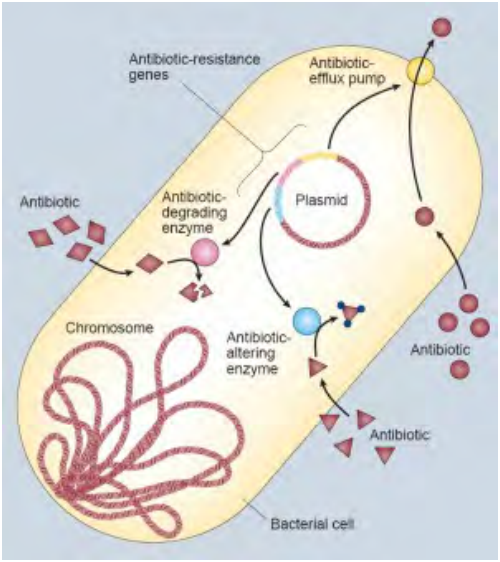

Figure 5. The causal cycle of antibiotic resistance. (From: CDC Threat Report 2013, http://www.cdc.gov/drugresistance/threat-report-2013/index.html)

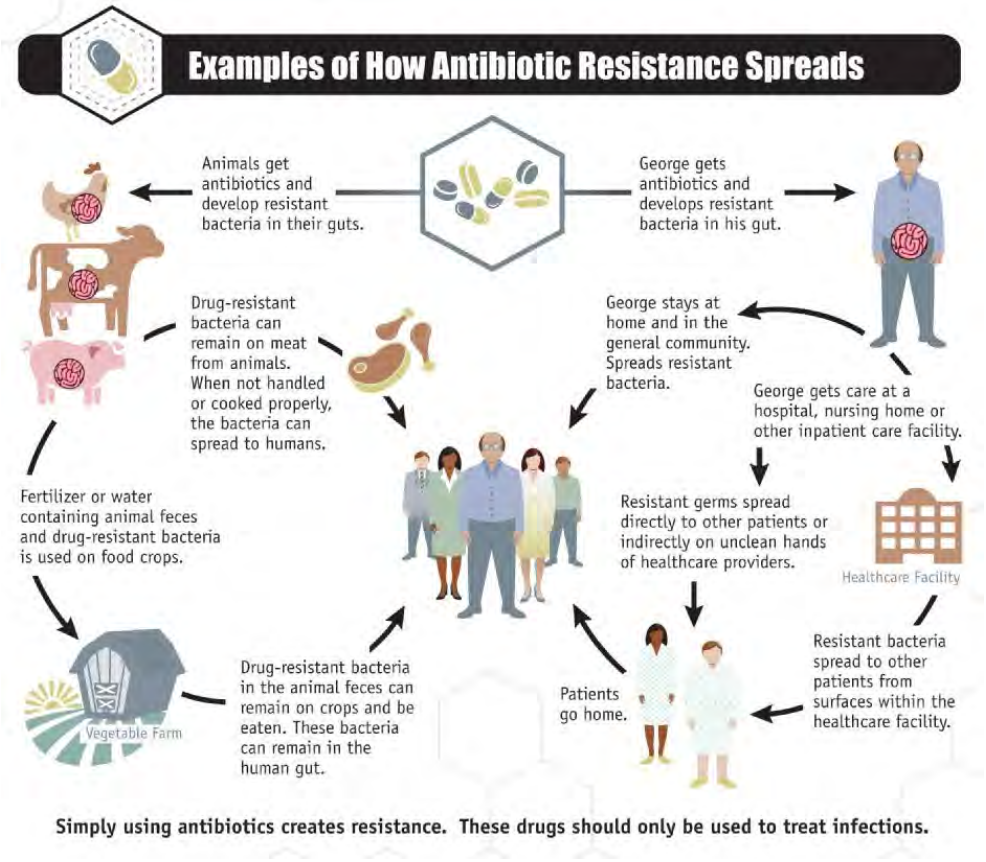

Figure 6. Cambodia and its neighbours in Southeast Asia (from http://www.mapsnworld.com/asia/south-east-asia-map-enlarge-view.jpg)

Figure 7. Cambodia geography and provinces.

Figure 8. Cambodia's health care facts at-a-glance. (adapted from Vlieghe E. et al., Journal of Global Antimicrobial Resistance 1 (2013) 31–34)

	Cambodia	Regional average**
Total population	14.805.000	
Size (km²)	181.035	
Gross national income per capita (PPP international $)	1870	9497
Total expenditure on health as % of GDP (2009)	5.8	
Human Development Index Ranking (out of 187 countries)	139	
Literacy rate (%)	74.4	82.2
Number of doctors (per 10.000 inhabitants)	2.3	14.5
Number of nurses/midwives (per 10.000 inhabitants)	7.9	20.3
Life expectancy at birth m/f (years)	57/65	72/77
Under five mortality rate (per 1 000 live births)	51	21
Access to improved water sources (average, %)	60	
Access to improved sanitation facilities (average, %)	30	
Proportion of communicable diseases among all deaths (%)	60	19
Prevalence of HIV (per 1000 adults, 15-49 years)	5	1
Prevalence of tuberculosis (per 100.000 population)	693	160

**sources: http://www.who.int/gho/countries/khm.pdf; http://www.unescap.org/stat/data/statind/pdf/t8_dec05.pdf; http://hdrstats.undp.org/en/countries/profiles/KHM.html;*

***WHO Western Pacific region*

Figure 9 Distribution of microbiology laboratories in Cambodia anno 2012. (adapted from Vlieghe E. et al., Journal of Global Antimicrobial Resistance 1 (2013) 31–34

1. Kantha Bopha Hospital (1992)
2. Institut Pasteur du Cambodge (1995)
3. NAMRU (2006)
4. National Institute of Public Health (NIPH) (2004)
5. MORU (Angkor Hospital for Children) (2005)
6. Sihanouk Hospital Center of Hope (2005)
7. Kampong Cham Hospital (2009)
8. Khmer-Soviet Friendship Hospital (2009)
9. Takeo Hospital (2010)
10. Battambang Hospital (2011)
11. National Pediatric Hospital (? 2011)
12. Kossamac Hospital (2011)
13. Calmette Hospital (2011)

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Chapter 2
What causes bloodstream infection in Cambodian adults?

Sub-culturing positive blood cultures in the microbiology laboratory of Sihanouk Hospital Centre of HOPE (Photograph by Jan Jacobs)

Bloodstream infection among adults in Phnom Penh, Cambodia: key pathogens and resistance patterns.

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Abstract

Background

Bloodstream infections (BSI) cause important morbidity and mortality worldwide. In Cambodia, no surveillance data on BSI are available so far.

Methods

From all adults presenting with SIRS at Sihanouk Hospital Centre of HOPE (July 2007 -December 2010), 20 ml blood was cultured. Isolates were identified using standard microbiological techniques; antibiotic susceptibilities were assessed using disk diffusion and MicroScan®, with additional Etest, D-test and double disk test where applicable, according to CLSI guidelines.

Result

A total of 5714 samples from 4833 adult patients yielded 501 clinically significant organisms (8.8%) of which 445 available for further analysis. The patients' median age was 45 years (range 15-99 y), 52.7% were women. HIV-infection and diabetes were present in 15.6% and 8.8% of patients respectively. Key pathogens included *Escherichia coli* (n=132; 29.7%), *Salmonella* spp. (n=64; 14.4%), *Burkholderia pseudomallei* (n= 56; 12.6%) and *Staphylococcus aureus* (n = 53; 11.9%). Methicillin resistance was seen in 10/46 (21.7%) *S. aureus*; 4 of them were co-resistant to erythromycin, clindamycin, moxifloxacin and sulphamethoxazole-trimethoprim (SMX-TMP).

We noted combined resistance to amoxicillin, SMX-TMP and ciprofloxacin in 81 *E. coli* isolates (62.3%); 62 isolates (47.7%) were confirmed as producers of extended spectrum beta-lactamase. *Salmonella* isolates displayed high rates of multidrug resistance (71.2%) with high rates of decreased ciprofloxacin susceptibility (90.0%) in *Salmonella* Typhi while carbapenem resistance was observed in 5.0% of 20 *Acinetobacter* sp. isolates.

Conclusions

BSI in Cambodian adults is mainly caused by difficult-to-treat pathogens. These data urge for microbiological capacity building, nationwide surveillance and solid interventions to contain antibiotic resistance.

Introduction

Sepsis is worldwide associated with important morbidity and mortality [1] with bloodstream infection (BSI) as one of its main causes. Early administration of adequate antibiotic therapy is essential to improve patient outcomes [2] and should be based on accurate knowledge of local bacterial pathogens and their resistance patterns. However, the worldwide emergence of antibiotic resistance has led to drastic changes in the choice of antibiotics for empirical or directed treatment in national or international treatment guidelines, with an evolution towards increased use of broad spectrum antibiotics.

Asia, and in particular its Southeast and eastern regions are struck profoundly by the problem of antimicrobial resistance [3]. Its extent has been described well in the regions' high and middle income countries, whereas accurate data and in depth research in low resources settings such as Cambodia are scarce.

Sihanouk Hospital Centre of HOPE (SHCH) is a Non-Governmental Organization (NGO) hospital for adults in Phnom Penh, Cambodia. It provides free care for the poor with specific focus on patients with the human immunodeficiency virus (HIV) and the chronically ill. Yearly, care is given to an average of 61.000 ambulatory and 1200 hospitalized patients. Microbiological facilities were installed in 2005, along with a local capacity building program focusing on diagnosis and management of antibiotic resistance at hospital level. In July 2007, a prospective blood culture study was initiated in patients presenting with presumed BSI.

The primary objective of this study was to determine the key bacterial pathogens and their resistance patterns causing invasive infections in Cambodian adults. As a secondary objective the information retrieved would be used for the redaction of locally adapted standard treatment guidelines. This would also include an exploration of possible risk factors for BSI and particular key pathogens. In this chapter we present the findings of this study.

Material and methods

Patient selection and microbiological methods

From all adult patients presenting at SHCH with signs of the Systemic Inflammatory Response Syndrome (SIRS)[4], venous blood (2 ×10ml) was drawn for culture along with registration of basic demographic and clinical data (including sex, age, duration of hospitalization, recent use of antibiotics, co-morbidity, presenting signs and symptoms and presumed focus of infection.)

Blood was cultured in home-made Brain Heart Infusion broth bottles (BIO-RAD, Hercules, US; 50 ml per bottle, July 2007-March 2009), and from April 2009 onward in BacTalert culture bottles (bioMérieux, Marcy l'Etoile, France). Blood cultures were incubated for 7 days at 35°C and daily monitored for growth by visual inspection of the broth or the chromogenic growth indicator where applicable. Isolates were stored at -70°C on porous beads in cryopreservative (Microbank, Pro-Lab Diagnostics, Richmond Hill, Canada). As part of the study protocol, isolates were retrieved and identification tests and antimicrobial susceptibility testing by disk diffusion were repeated at the Institute of Tropical Medicine, Antwerp (ITM, Belgium).

Isolates were identified using standard microbiological techniques and Micro**S**can®(Combo 42, Siemens Healthcare Diagnostics, Deerfield, USA). *Salmonella* spp. were serotyped according to the Kauffman-White schedule[5]. Antibiotic susceptibilities were assessed by disk diffusion (Neo-Sensitabs™, Rosco Diagnostica, Taastrup, Denmark) and Micro**S**can®. Additional testing, where applicable, included Etest (bioMérieux, for determination of penicillin minimal inhibitory concentrations (MIC) in *Streptococcus pneumoniae*), D-test [6] for the detection of inducible clindamycin resistance in *Staphylococcus aureus* and double disk test with ceftazidime, cefepime, ceftazidime-clavulanic acid and cefepime-clavulanic acid as described elsewhere[7] (for extended spectrum beta-lactamase-screening in *Enterobacteriaceae*). Interpretive criteria were those defined by the Clinical and Laboratory Standards Institute [8]. For azithromycin and *Enterobacteriaceae*, no breakpoints have been published. EUCAST mentions treatment of *Salmonella* Typhi infections with a MIC≤ 16 µg/mL and a recent publication proposed 16 µg/ml as 'epidemiological cut-off' value for wild type *Salmonella* spp [9]. The following isolates were considered as contaminants: coagulase-negative staphylococci, *Bacillus* spp., *Corynebacterium* spp. Non-eubacterial pathogens (*e.g.* mycobacteria and fungi) were left out of the analysis.

The minimum number of isolates per species for separate antibiotic resistance reporting was set at 10, in line with the recommendations of the ESCMID Study Group for Antimicrobial Resistance Surveillance (ESGARS)[10] and of the U.S. Clinical and Laboratory Standards Institute (CLSI) at the time of the initiation of the study (*i.e.* CLSI M2-18).

For the assessment of antimicrobial susceptibility rates, only the first isolate per patient was considered. Growth from a repeat blood culture sampled more than 14 days after the previous one in a patient on adequate antibiotic treatment was considered as a new BSI episode. Recurrent isolates (*i.e.* same species isolates from different BSI episodes) and consecutive isolates (*i.e.* different species' isolates from different BSI episodes) were not compiled in the resistance overview.

Isolates from patients who were not hospitalized or admitted no more than two days on the moment of blood culture sampling were considered community-acquired whereas isolates from patients hospitalized longer than two days were considered hospital-acquired. 'Shock' was defined as presenting with a mean arterial pressure (MAP) of < 60 mmHg despite adequate intravenous fluid resuscitation [4]. Fever and cough were considered 'acute' if less than three weeks of duration and 'chronic' if persisting for ≥ 3 weeks. The period between November 16th and May 15th was considered the dry season, and May 16th- November 15th the wet season.

Data registration and statistical analysis

Data were entered in Access and Excel (Microsoft Corporation, Redmond, Washington, USA). Stata software, version 11.2. (Stata Corporation, College Station, TX, USA) was used for statistical analysis. Risk factors for BSI and particular key pathogens were explored using univariate analysis (chi-square tests). Differences were considered statistically significant at p-values < 0.05.

Ethical approval

Ethical approval was granted from the review boards at the Institute of Tropical Medicine (ITM), Antwerp, the University Hospital Antwerp and the National Ethical Committee, Phnom Penh, Cambodia respectively. A waiver for prior informed consent was obtained since samples were taken as part of routine clinical care. Patients were identified with a unique hospital number. For the clinical and epidemiological data, no other data besides those noted in the routine medical files were used.

Results

Demographical and epidemiological data

Between July 1st 2007 and December 2nd 2010, a total of 4833 adult patients with SIRS attended the hospital and had blood cultures drawn during 5714 SIRS episodes (Figure 1). Fifty-three per cent of the patients were women, with a median age of 45 years (15-99 y, Table 1). Patients came from at least 14 different provinces in Cambodia, predominantly from the Central and South of the country. Co-morbidity was noted in nearly one third (1579/4833; 32.7%) of the patients, mainly HIV-infection (15.6%) or diabetes mellitus (8.8%). Eighty-six per cent (4912/5714) of the SIRS episodes were community-acquired, while 370 of them (6.5%) were considered hospital-acquired. Recent use of antibiotics prior to blood culture sampling was noted in 1270 episodes (22.2%).

Acute fever (n = 4117; 72.1%) was the most common presenting symptom followed by acute cough (n = 445; 7.8%), chronic fever (n = 211; 3.7%), diarrhoea (n = 196; 3.4%), chronic cough (n = 176; 3.1%), dysuria (n = 176; 3.1%), meningism (n = 140; 2.5%) and shock (n = 100; 1.8%). Abdominal, respiratory and urogenital infections were the most common presumed foci of BSI (Table 1).

Microbiological data

In four hundred and sixty-three SIRS episodes, 501 clinically significant organisms (CSO) were cultured (yield 8.8%). In addition, a total of 496 contaminants (contamination rate 8.7%) were isolated. The CSO yield in samples from patients with prior antibiotic use (112/1270; 8.8%) was not different from patients without known antibiotic use (389/4440, 8.8%).

A total of 445 CSO were available for further analysis. Of those, 404 (90.8 %) were isolates from community-acquired infections and 41 (9.2%) were hospital-acquired.

As shown in Table 2, Gram-negative pathogens were predominant (351/445; 78.9%). The most frequent pathogens overall included *Escherichia coli* (n=132), *Salmonella* spp. (n=64), *Burkholderia pseudomallei* (n=56) and *Staphylococcus aureus* (n = 53). Hospital-acquired BSI were mainly caused by *S. aureus* (5/41; 12.2%), *E. coli* and other *Enterobacteriaceae* (21/41; 51.2%) and *Acinetobacter* spp. (7/41; 17.1%). Upon retesting at ITM, we observed genus identification agreement in 98.4% of isolates; a particular species identification disagreement was noted for the first isolates of *Streptococcus suis* (n = 3) and *Salmonella* Choleraesuis (n = 17).

In 426 patients a single BSI episode was noted whereas nineteen patients experienced multiple episodes of BSI (Figure 1). Of those patients, thirteen had genuine recurrent BSI (*i.e.* with the same species), most commonly caused by *Salmonella* Choleraesuis (a total of 17 episodes in seven patients), *S. aureus* (seven episodes in three patients) and *E. coli* (three patients with two episodes each). Six other patients had consecutive episodes of BSI (*i.e.* with different species). Only the isolates of the first BSI episodes were taken into account for the calculation of the key pathogens' resistance rates (Table 2).

BSIs were generally more frequent in patients with diabetes (59/494 (11.9%) versus 442/5220 (8.5%) patients without diabetes, p = 0.009) and during the wet season (299/3132 (9.5%) versus 202/2582 (7.8%) episodes during the dry season, p = 0.020) (Figure 2). *E coli* BSI occurred more frequently in patients aged ≥ 60 years (37/1066 (3.5%) versus 90/4567 (2.0%) patients < 60 years old, p =0.003) and in those with diabetes (20/494 (4.0%) versus 112/5220 (2.1%) patients without diabetes, p = 0.007), whereas *B. pseudomallei* BSI was seen more often in men (40/2685 (1.5%) versus women

16/3006 (0.5%), p <0.005) and diabetes patients (14/494 (2.8%) versus 42/5220 (0.8%) in those without diabetes, p < 0.005). In contrast, *Salmonella* BSI occurred more often in HIV-positive patients (37/1186 (3.1%) versus 30/4528 (0.7%) HIV-negative patients, p < 0.005) as was also the case for *S. aureus* BSI (17/1186 (1.4%) HIV-positive patients versus 36/4528 (0.8%) HIV-negative patients, p =0.041).

Out of 501 BSI patients, 330 (65.9%) recovered, 113 died (22.5%), 25 (4.9%) were referred to another hospital, 33 (6.6%) had an unknown outcome. The median outcome follow-up time was 138 days (range 0-1982 d); death occurred after a median duration of 2 days of BSI diagnosis (range 0-62 d). Mortality was higher than average in patients with BSI due to *B. pseudomallei* (29/55; 52.7%) and *E. coli* (35/131; 26.7%), and lower in those with BSI due to *S. aureus* (9/53; 17%) and *Salmonella* spp. (17/66; 10.6%). In addition, we found higher mortality rates in patients with BSI due to third generation-cephalosporin resistant *E. coli* (20/50 (40.0%) versus 15/65 (23.1%) in those without this resistance pattern) and for patients with methicillin susceptible *S. aureus* (MSSA, 8/38 (21.1%) versus those with methicillin resistant *S. aureus* (MRSA) BSI (1/8; 12.5%), but both findings were not statistically significant. Prior use of antibiotics influenced significantly the presence of third generation-cephalosporin resistance in *E. coli* BSI (15/19 (79.0%) versus 46 of 112 (41.9%) patients without prior antibiotic treatment; p = 0.002), but it did not significantly affect the overall patients' mortality (21/103 (20.4%) versus 92/340 (27.1%) in those without prior antibiotic use; p = 0.20).

Gram-positive cocci

Table 3 displays the antibiotic resistance of methicillin resistant *S. aureus* (MRSA) and methicillin susceptible *S. aureus* (MSSA). MRSA was present in 10 of 46 *S. aureus* (21.7%) from first BSI episodes and was associated with high levels of resistance to clindamycin, moxifloxacin, erythromycin and tetracycline. Four MRSA isolates (25.0%) displayed combined resistance to erythromycin, clindamycin, SMX-TMP, moxifloxacin and tetracycline. This co-resistance was also noted to a lesser extent in MSSA (2/36 isolates (5.6%)). Glycopeptide susceptibility was preserved. The presence of methicillin resistance in *S. aureus* BSI was not significantly more prevalent in patients with prior antibiotic use (3 of 12 (25.0%) than among those without antibiotic pre-use (7/41 (17.0%), p = 0.70). In the 11 *S. pneumoniae* isolates, a median MIC penicillin of 0.12 µg/ml (range 0.012 - 4 µg/ml) was noted, which translates according to the 2012 CLSI guidelines [8] as intermediate and high level resistance to oral penicillins in 5 and 2 isolates respectively, and as intermediate resistance to parenteral penicillins in one isolate for non-meningitis patient. Resistance for SMX-TMP, ceftriaxone, and erythromycin was noted in 8/11 (72.7%), 0/11 (0%) and 2/11 (18.2%) of the isolates.

Enterobacteriaceae

As shown in Table 4, *E. coli* and other *Enterobacteriaceae* were extensively resistant to all commonly available oral first line antibiotics (*i.e.* ampicillin, SMX-TMP and ciprofloxacin) and gentamicin. Nearly half of all *E. coli* (47.7%) were confirmed extended spectrum beta-lactamase (ESBL) producers. *Enterobacter* spp. displayed also considerable resistance rates for reserve antibiotics (*e.g.* piperacillin-tazobactam, tigecyclin, colistin, amikacin).

In addition, one third of all *Enterobacteriaceae* (58/175) were co-resistant for the most commonly available oral or parenteral antibiotics in Cambodia (*i.e.* ampicillin, SMX-TMP, ciprofloxacin, gentamicin and third generation cephalosporins). Co-resistance to amikacin was noted in 3 of 175 (1.7%) isolates (Table 5).

The resistance patterns of *Salmonella* spp. from this BSI study were described separately[11]. We observed high rates of multidrug resistance (*i.e.* co-resistance to ampicillin, SMX-TMP and chloramphenicol as described by Rowe et al, Clin Infect Dis 1997, 24: S 106-7) in 75.0% of *Salmonella* Typhi, 91.7% of *Salmonella* Choleraesuis and in 38.5% of other NTS . Decreased ciprofloxacin susceptibility was very common in *Salmonella* Typhi (14/15 isolates, 90.0%) and somewhat less frequent in *Salmonella* Choleraesuis (20.8%) and other NTS (53.8%) while azithromycin resistance was very common in *Salmonella* Choleraesuis (17/24 isolates, 70.8%) and emerging in other NTS (15.4%) and *Salmonella* Typhi (5.0%). Two *Salmonella* Choleraesuis isolates were extended spectrum beta-lactamase producers. One NTS isolates displayed high level ciprofloxacin resistance.

In addition to their natural resistance for ampicillin, most *Aeromonas* isolates were largely resistant for amoxicillin-clavulanic acid (10/11; 90.9%) and to a lesser extent for cefotaxime (1/11; 9.0%), SMX-TMP and meropenem (2/11, 18.2% each) but they remained fully susceptible for ciprofloxacin.

Non-fermentative Gram-negative rods

As we described elsewhere [12], we did not observe *Burkholderia pseudomallei* isolates resistant for ceftazidime, meropenem, amoxicillin/clavulanic acid or doxycycline, but 12 (22.2%) isolates had MIC's equal to the susceptibility breakpoint for chloramphenicol. *B. pseudomallei* has intrinsic resistance for amoxicillin, aminoglycosides and polymyxins while fluoroquinolones have only weak clinical efficacy [13].

High resistance levels for most available effective drugs and emerging carbapenem resistance were observed in other non-fermentative Gram-negative rods (*i.e.* particularly *Acinetobacter* and *Pseudomonas* spp. (Table 6). Of note, *Acinetobacter* sp. was found to be of hospital-acquired origin in at least 7of 20 (35%) isolates.

Discussion

In our study we observed a predominance of Gram-negative pathogens causing BSI in Cambodian adults; the three most frequent pathogens were *E. coli*, *Salmonella* spp., *B. pseudomallei*, followed by *S. aureus*. Many of these pathogens showed resistance to the antibiotics which are commonly available in Cambodia either by natural or by acquired resistance (*e.g. B. pseudomallei* and ESBL-producing bacteria respectively). In particular, resistance rates are alarming in *Enterobacteriaceae* displaying third generation cephalosporin resistance in up to 50% of isolates and nearly 1 in 3 isolates displaying complex combined resistance leaving very few treatment options.

One of the primary strengths of our findings is the fact that they were derived from systematically collected bloodstream isolates and obtained through a 'real live' capacity building program, where we observed excellent identification correlations between the laboratories of SHCH and ITM.

For the local health care workers, these data functioned as an 'eye opener' regarding the incidence in Cambodia of certain pathogens and specific resistance patterns (*e.g. B. pseudomallei*, ESBL-positive *Enterobacteriaceae*, *Streptococcus suis*, *Salmonella* Choleraesuis). They were also the basis of a better communication between the laboratory and the clinicians at SHCH and initiated a set of antibiotic stewardship activities. These included educational sessions, the development and implementation of standard treatment guidelines, the creation of a hospital essential drug list, enhanced communication with antibiotic donation agencies and finally collaboration with initiatives at national level.

Our study had also several limitations. First, the high rates of co-morbidity in our study population may have caused over-representation of specific pathogens (*e.g. B. pseudomallei* in diabetic patients, *Salmonella* Choleraesuis in patients with HIV). Therefore these findings should be interpreted in the context of these specific patient groups. However, given the fast evolution of the Asian diabetes epidemic and the ongoing HIV-epidemic, rising numbers of these particular invasive bacterial infections may be expected.

Likewise, the widespread use of antibiotics in the community prior to attendance at the hospital (as noted in a quarter of the patients with confirmed BSI) may have led to underrepresentation of more fastidious organisms (*e.g. S. pneumoniae*) and/or the selection of the more resistant organisms, although we did not observe any differences in CSO yield between patients with and without prior antibiotic use. Furthermore, the distinction between community- and hospital-acquired infection

was not always crystal clear as patients may not have always mentioned their prior visits to health care centres or private clinics. Therefore our findings may not fully represent the pathogen distribution and resistance patterns in the general population; other methodologies have been suggested for this purpose [14].

In addition, it was not among the present objectives to include media for mycobacterial blood culture nor additional testing for other likely causes of fever in Cambodia *e.g.* influenza, malaria, leptospirosis, dengue, scrub typhus. In this respect our data complement other research [15] on the causes of fever in Cambodian patients attending first line health services.

Finally, clinical and epidemiological data were not available from all patients and, along the isolation of CSO, we observed a high contamination rate of the blood cultures. As part of a larger capacity-building project, considerable effort was spent to tackle this problem with education and feedback sessions.

In spite of these limitations, we think that our findings are well in line with and are complementing other data from the southeast Asian region. For instance, blood culture studies from Thailand [16, 17] also described a high prevalence of Gram-negative pathogens, with a predominance of non-typhoid *Salmonella* in HIV-positive patients. Besides *B. pseudomallei*, also *Streptococcus suis* [18] and *Salmonella* Choleraesuis [19] appear to be regionally relevant pathogens. In addition, *Acinetobacter* spp. has been described also a common cause of hospital- and community-acquired invasive infections in other Asian settings [20] as well. The relative paucity of *Salmonella* Typhi in our study is probably due to the fact that the hospital has no paediatric services and attracts a somewhat older and comorbid population.

Recent surveillance data from the Asian region [21] show overall high prevalence of ESBL in *Enterobacteriaceae*. The nearly 50% ESBL prevalence among *E. coli* in our study appears well in line with the rates of ESBL–positivity of this pathogen in Thailand (56%) and Vietnam (42.0%). Only in India and China the prevalence of ESBL in *E. coli* was higher (67.1 and 65.4% respectively).

The presence of ESBL in *E. coli* and other *Enterobacteriaceae* in Southeast Asia is a relatively recent phenomenon, its presence and exponentially rising prevalence occurred in the literature around the start of the new millennium [22]. Of note, much lower resistance rates were noted in Laos [23], *i.e.* 8% and 33% ceftriaxone resistance in *E. coli* and *K. pneumoniae* respectively.

As our report provides the first description of ESBL-positive pathogens from systematically collected community-acquired bloodstream infections, it complements the observations of Ruppé and coworkers [24] in 2007, describing the presence of ESBL in 37.7% of *E. coli* from 93 urinary samples

from patients in Phnom Penh, all of the CTX-M- type. In depth molecular studies of the ESBL-types in the *Enterobacteriaceae* from our study will be further described in Chapter 5.

So far we did not yet observe carbapenem resistance in *Enterobacteriaceae* in Cambodia, but this may only be a matter of time given the recently found presence of NDM-1 positive *Enterobacteriaceae* in seepage water in Hanoi, Vietnam [25] and in clinical samples in Thailand [26]. In addition, given the high ESBL-rates, carbapenem antibiotics have been recently introduced in the country. Finally, we found carbapenem resistance in nearly 5% of *Acinetobacter* sp., which may provide an additional reservoir of resistance genes.

Our data highlighted also *S. aureus* as an important cause of BSI in Cambodia. This confirms findings from other Southeast Asian countries *e.g.* Laos and Thailand [27]; the Thai authors observing comparable MRSA rates. Our data on *S. aureus* BSI complement the recent descriptions of MRSA as a cause of community-acquired skin and soft tissue infections in children in Siem Reap (Northwest Cambodia)[28].

What may be the main drivers of these high resistance rates in Cambodia, a country that has just recovered from many years of civil war and international isolation? However speculative, we assume that the unregulated use of antibiotics in men and livestock plays a pivotal role. While solid usage data for antibiotics in Cambodia are absent so far, there is a large body of published information on poor quality anti-parasitic drugs (in particular antimalarials) in Cambodia and in the Southeast Asian region [29]. Further, a pilot survey in three Cambodian health centres [30] revealed a 66-100% antibiotic prescription rate per consultation, with antibiotic prescriptions being appropriate in as low as 3-45% of cases. Recent agricultural reports from neighbouring Vietnam [31] and Thailand showed long lists of antibiotics used for the prevention and treatment of infections in livestock, including fluoroquinolones, beta-lactams, cephalosporins, macrolides and even actual third line antibiotics such as polymyxins. A recent Cambodian study on 152 chicken carcasses collected at 10 markets across the country revealed high contamination rates with enteric pathogens, many of which with complex resistance patterns [32]. Taken together, these facts suggest intense antibiotic use in humans and animals in the entire region which warrants urgent surveillance and regulation.

Further transmission of selected resistant organisms may then occur at crowded homes with insufficient sanitation or potable water supply, or in health care settings where basic infrastructure and infection control measures are often lacking or not seen as a priority [33]. Nosocomial infection in low-resource settings is probably frequent but underreported [34].

As a response, Cambodia's Health authorities issued a first National Medicines Policy in Cambodia in 1995; a more extensive Pharmaceutical Sector Strategic Plan was introduced in 2006 with the aim of promoting Good Pharmacy Practice while focusing on access, quality and rational use of drugs [35]. In addition, a comprehensive plan for the improvement of infection control at the health care facilities was issued in 2010 [36].

If confirmed in other settings in the country, the observed high resistance rates seriously jeopardize the treatment options for community-associated BSI in Cambodia. Based on these microbiological data, over one third of individuals with BSI in SHCH would require treatment with broad spectrum antibiotics which are not available in most public hospitals in Cambodia, nor included in the actual national essential drugs list (with the exception of ceftazidime)[37]. In SHCH broad spectrum antibiotics such as ceftazidime, meropenem and vancomycin were obtained via a drug donation system, integrated in the local standard treatment guidelines and their use is monitored. This is however not yet the case for most other health care settings in the country. Given the increased morbidity and mortality associated with the use of ineffective empirical antibiotics for invasive infections with resistant bacteria [2], as also suggested by the outcome data in our study, there is an urgent need for sustainable and affordable access to third line antibiotics for the treatment of ESBL-positive *E. coli* and other *Enterobacteriaceae*, melioidosis and invasive MRSA infections in the entire country. Along such an updated essential drugs list, standard treatment guidelines and the medical curriculum require thorough and urgent revision. This is however a lengthy process and holds important financial and organisational implications. In addition, locally adapted antibiotic stewardship and infection control programs should be introduced at the different levels of patient care, while nationwide surveillance of bacterial resistance and antibiotic use is essential for planning and monitoring of these interventions. Ideally, this resistance problem should be addressed and surveyed at a regional level. Finally, the introduction of targeted vaccination *e.g.* for typhoid fever, *S. pneumoniae* and *Haemophilus influenzae* type B (HiB) may be a complementary intervention which has proven successful in reducing the number of infections in other low-resource settings[38].

Confirmation of our findings from blood cultures studies in rural hospitals and other patient groups in Cambodia (*e.g.* paediatric and adolescent patients) is certainly needed as well as community-based surveillance of resistance in healthy carriers. An expansion of solid microbiological capacity in Cambodia is urgently needed to ensure these clinical, research and surveillance activities.

Further research would also include quantitative and qualitative research on the use of antibiotics in the community, health care settings and agriculture and further identification of those most at risk

of life-threatening resistant bacterial infections, *e.g.* the elderly, patients with diabetes or HIV as suggested by our exploratory findings.

Conclusion

Adult patients with community-acquired BSI in Cambodia are very likely to be infected with highly resistant Gram-negative pathogens. These findings warrant intensified microbiological surveillance and should be a an urgent call for concerted nation-wide action to contain bacterial resistance in Cambodia.

Figures and tables

Figure 1. Flow chart of patients with SIRS episodes and corresponding episodes of BSI.

(SIRS was defined as the presence of more than one of the following clinical findings: body temperature of > 38°C or < 36°Celsius, heart rate > 90 beats per minute, respiratory rate > 20 per minute, $PaCO_2$ < 32 mmHg, white blood cell count > 12000 cells/µL or < 4000 cells /μL^4.)

Figure 2. Monthly number of patients with culture-confirmed BSI, SHCH 2007-2010

Table 1. Demographic and clinical characteristics of patients with SIRS and BSI (SHCH, 2007-2010)

Table 2. Pathogen distribution in 445 blood culture isolates, SHCH 2007-2010.

Table 3. Antibiotic resistance patterns of 46 *S. aureus* from blood, SHCH 2007-2010.

Table 4. Antibiotic resistance patterns of *Enterobacteriaceae* from blood, SHCH 2007-2010.

Table 5. Combined resistance in *Enterobacteriaceae* from blood, SHCH 2007-2010.

Table 6. Antibiotic resistance patterns of non-fermentative Gram-negative rods from blood, SHCH 2007-2010.

Figure 1. Flow chart of patients with SIRS and bloodstream infection (BSI), SHCH 2007-2010.

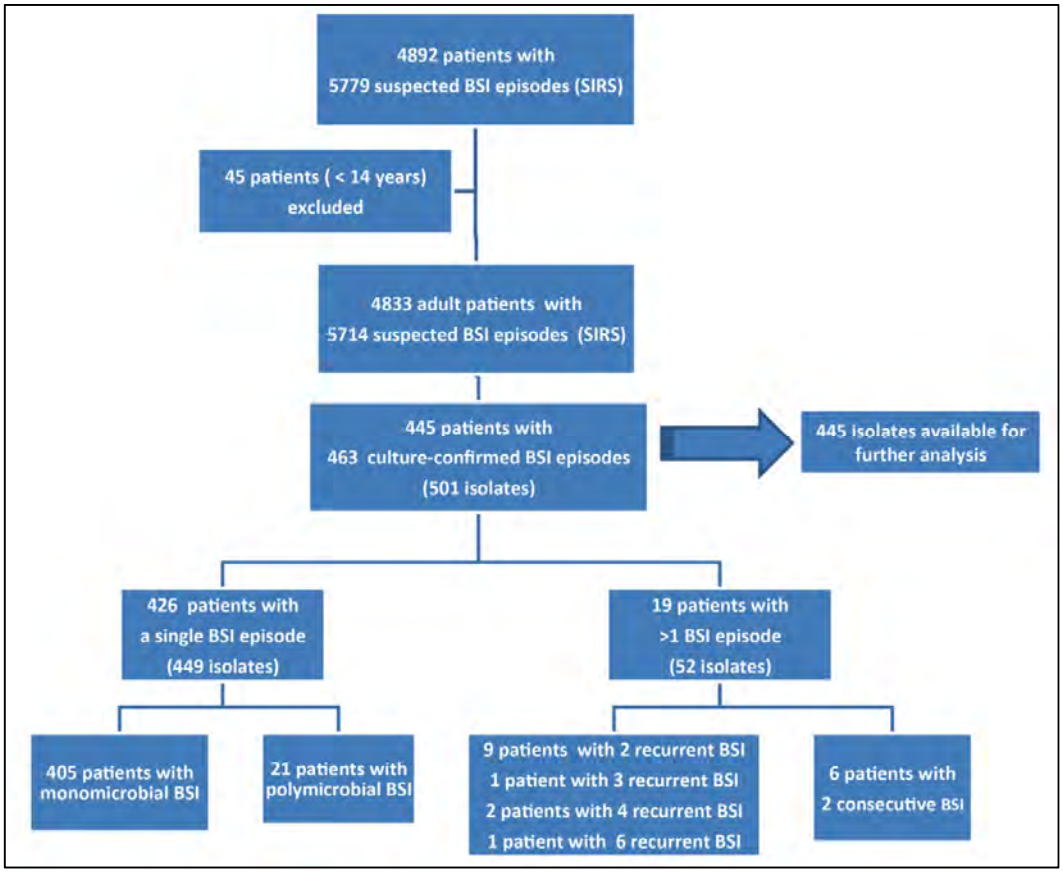

Figure 2. Monthly number of BSI in SHCH, 2007-2010

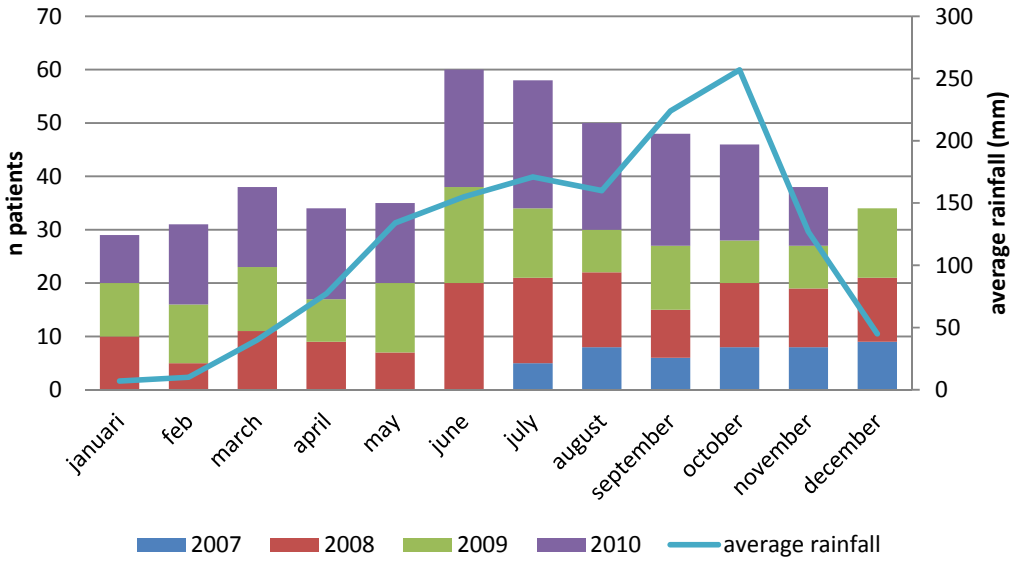

Table 1. Demographic and clinical characteristics of patients with SIRS and BSI (SHCH, 2007-2010)

		all SIRS-episodes		culture confirmed BSI-episodes	
		n	%	n	%
Total number of patients		4833		445	
Female		2547	52.7	226	50.8
Age (range)		45 (15-99)		46 (15-96)	
Co-morbidity	HIV-positive	755	15.6	82	18.4
	diabetes	425	8.8	51	11.5
	liver cirrhosis	196	4.1	25	5.6
	other*	286	5.9	23	5.2
Total number of episodes		5714		463	
Hospitalisation**	Not or ≤ 2 days	4912	86.0	404	87.3
	> 2 days	370	6.5	48	10.4
	No data	423	7.4	46	9.9
Recent antibiotic treatment		1270	22.2	112	24.2
Presumed focus***	abdominal	2049	35.9	190	41,0
	respiratory	1909	33.4	139	30,0
	urogenital	576	10.1	57	12,3
	SSTI	480	8.4	43	9,3
	meningeal	348	6.1	30	6,5
	osteoarticular	84	1.47	6	1,3
	deep organ abscess	15	0.26	3	0,6
	disseminated infection	659	11.5	59	12,7
	unknown	1310	22.9	116	25,1

SIRS: systemic inflammatory response syndrome; BSI: blood stream infection; HIV: human immune deficiency virus; SSTI: skin and soft tissue infections

** includes chronic lung or renal disease, chronic use of steroids; **refers to duration of hospitalisation at moment of blood culture sampling; ***in some patients more than one presumed focus was noted*

Table 2. Pathogen distribution in 445 blood culture isolates, SHCH 2007-2010.

Species	total n (%)	n HA isolates*	n first BSI's isolates**
Gram-positive cocci	94		
Staphylococcus aureus	53 (11.9)	5	46
β-hemolytic streptococci group A	2 (0.4)	-	-
group B	2 (0.4)	-	-
group C	4 (0.9)	-	-
group G	1 (0.2)	-	-
Streptococcus pneumoniae	11 (2.5)	2	11
Streptococcus suis	9 (2.0)		-
Streptococcus anginosus group	2 (0.4)	1	-
Streptococus bovis	2 (0.4)	-	-
Streptococcus salivarius	2 (0.4)	-	-
Enterococci spp.	6 (1.3)	-	-
Gram-negative bacilli	351		-
Enterobacteriaceae			-
Escherichia coli	132 (29.7)	13	130
Klebsiella pneumoniae	34 (7.6)	5	32
Enterobacter spp.	14 (3.1)	3	13
Proteus spp.	5 (1.1)	-	-
Aeromonas spp.	11 (2.5)	-	11
Shigella spp.	2 (0.4)	-	-
Vibrio cholerae	1 (0.2)	-	-
Citrobacter sp.	1 (0.2)	-	-
Salmonella Typhi	15 (3.4)	1	15
Salmonella paratyphi A	2 (0.4)	-	-
non -typhoid *Salmonella* spp. (NTS)		-	-
serovar Choleraesuis	35 (7.9)	1	23
serovar Enteritidis	6 (1.3)	-	-
serovar Typhimurium	4 (0.9)	-	-
other NTS	2 (0.4)	1	-
non-fermentative Gram-negative rods	87	-	-
Burkholderia pseudomallei	56 (12.6)	1	56
Acinetobacter spp.	20 (4.5)	7	20
Pseudomonas spp.	8 (1.8)	-	8
other	3 (0.7)	1	-
Total	445	41	365

** HA: hospital acquired isolates;** isolates used for calculation of resistance rates*

Table 3. Antibiotic resistance patterns of 46 *S. aureus* from blood, SHCH 2007-2010.

Antibiotic	MRSA (n = 10)	MSSA (n = 36)
Penicillin	100	97.2
Erythromycin	90.0	41.7
Clindamycin*	100	38.9
Tetracycline	90.0	41.7
SMX-TMP	60.0	13.9
Moxifloxacin	90.0	30.6
Vancomycin	0.0	0.0
Fusidic acid	10.0	5.6

**including inducible clindamycin resistance, SMX-TMP: sulphamethoxazole-trimethoprim; MRSA: methicillin resistant Staphylococcus aureus; MSSA: methicillin susceptible Staphylococcus aureus*

Table 4. Antibiotic resistance patterns of *Enterobacteriaceae* from blood, SHCH 2007-2010.

Antibiotic	*E. coli* (n = 130)	*Klebsiella* spp. (n = 32)	*Enterobacter* spp. (n = 13)
	% resistant isolates		
Ampicillin	93.8	100,0	100,0
Amoxicillin/clavulanic acid	49.2	28.1	84.6
SMX-TMP	95.4	75.0	76.9
Ciprofloxacin	65.4	21.9	46.2
Cefotaxime	51.5	46.9	61.5
Ceftazidime	36.2	25.0	46.2
Cefepime	46.2	28.1	46.2
ESBL confirmed*	47.7	43.8	46.2
Gentamicin	56.2	28.1	38.5
Amikacin	3.8	0.0	7.7
Piperacillin/tazobactam	9.2	15.6	23.1
Meropenem	0.0	0.0	0.0
Colistin	0.8	3.1	53.8
Tigecycline	0.0	6.3	15.4

** by double disk testing; SMX-TMP: sulphamethoxazole-trimethoprim; ESBL: extended spectrum β-lactamase*

Table 5. Combined resistance in *Enterobacteriaceae* from blood, SHCH 2007-2010.

	E. coli	*Klebsiella* spp.	*Enterobacter* spp.
	(n = 130)	(n = 32)	(n = 13)
Antibiotic	% resistant isolates		
Ampicillin + SMX-TMP + ciprofloxacin (AmSxCip)	62.3	18.8	46.2
AmSxCip + gentamicin	47.7	12.5	30.8
AmSxCip + gentamicin + cefotaxime	38.5	12.5	30.8
AmSxCip + gentamicin + cefotaxime + amikacin	2.3	0.0	0.0

SMX-TMP: sulphamethoxazole-trimethoprim

Table 6. Antibiotic resistance patterns of non-fermentative Gram-negative rods from blood, SHCH 2007-2010.

	Acinetobacter spp.	*Pseudomonas* spp.
Antibiotic	(n = 20)	(n = 8)
SMX-TMP	70.0	-
Ciprofloxacin	45.0	12.5
Gentamicin	50.0	0.0
Amikacin	35.0	0.0
Ceftazidime	45.0	0.0
Cefepime	45.0	12.5
Piperacillin/tazobactam	0.0	0.0
Meropenem	5.0	12.5
Colistin	15.0	12.5

SMX-TMP: sulphamethoxazole-trimethoprim;

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Chapter 3
Melioidosis, an unknown killer

Bilateral pneumonia is a common and life-threatening presentation of melioidosis in Cambodian adults. Out of the 28 patients presenting with pulmonary melioidosis in SHCH between 2007 and 2010, 18 (64.3%) died, especially those who had also BSI and patients who received inappropriate empirical therapy.

Melioidosis in Cambodia: clinical and microbiological data in 58 adult patients.

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Abstract

Melioidosis, an infectious disease caused by the Gram-negative pathogen *Burkholderia pseudomallei*, is a common cause of community-acquired bloodstream infection and pneumonia in northern Australia, Thailand and other areas in Southeast Asia. Data on its presence and presentation in Cambodia are scarce. We describe the clinical presentation, outcome and resistance patterns in patients diagnosed with melioidosis during a prospective surveillance study of febrile adult patients attending Sihanouk Hospital Centre of HOPE, Phnom Penh, Cambodia.

Between July 1st 2007 and January 31st 2010, 58 patients were diagnosed with melioidosis in SHCH. They had a mean age of 49 years (range 18-73); 58.6% were male. Risk factors, seasonality and clinical presentation were in line with findings from neighboring countries. Melioidosis presented as bloodstream infection (BSI) in 39 (67.2%) patients *i.e.* 12.0 % of all BSI's during the study period. Mortality was high (51.7%; 30/58) especially in those presenting with BSI (RR 6.8 (1.82-25.5), $p<0.001$), with shock or multiple organ failure (RR 4.59 (1.60-13.32), $p < 0.001$) and patients receiving inappropriate empirical therapy (RR 3.5 (2.07-5.90), $p < 0.001$). Patients with limited infection in skin and soft tissue had better prognosis than average (RR 0.48 (0.24-0.97), $p = 0.023$).

The empirical treatment was appropriate in 35 of 58 patients and included mostly ceftazidime + sulfamethoxazole-trimethoprim (SMX-TMP) followed by a maintenance treatment of SMX-TMP + doxycyclin. Improving outcomes will require faster diagnostic methods, the availability of broad spectrum antibiotics and improved sepsis care.

Background

Melioidosis is a severe community-acquired disease caused by *Burkholderia pseudomallei*, endemic in Southeast Asia and tropical Australia [1, 2]. It presents as lung or soft tissue infections with or without bloodstream infection (BSI) [3] and is subject to regional and seasonal variation [4]. In severe cases, mortality can exceed 80%, and relapse rates are high. Acute infections require treatment with third generation cephalosporins or carbapenems, followed by maintenance courses of sulfamethoxazole-trimethoprim (SMX-TMP) with or without doxycyclin. With regards to Cambodia, serological evidence has been published earlier [5] and few microbiologically confirmed cases have been described [6-8] including a series of pediatric cases [9].

Sihanouk Hospital Centre of Hope (SHCH), a 40-bed Non-Governmental Organization (NGO) referral hospital in Phnom Penh provides free care for poor adults. Care is given to a yearly average of 61.000 ambulatory and 1200 hospitalized patients. The hospital started in 2007 with systematic surveillance of BSI. Within the first months, *B. pseudomallei* was isolated from several patients. In this chapter we describe the clinical and microbiological characteristics of the first 58 patients with culture-proven melioidosis admitted to SHCH.

Material and methods

Patient selection and microbiological methods

Melioidosis was defined on microbiological criteria, *i.e.* growth of *B. pseudomallei* from any clinical specimen [3].

Blood cultures were taken as specified in Chapter 2. Over the two year study period, clinical work-up of other clinical samples such as sterile body fluids, pus and urine cultures was introduced.

Isolates were withheld as *Burkholderia pseudomallei* when they presented as non-fermentative Gram-negative rods showing bipolar staining, wrinkled colony aspect on blood agar plate, oxydase positive, polymyxin and gentamicin resistant and susceptible to amoxicillin-clavulanic acid [11] (disk diffusion, Rosco, Taastrup, Danmark). They were further identified with the API 20 NE system (bioMérieux), France.

Isolates were stored at -70°C on porous beads in cryo-preservative (Microbank, Pro-Lab Diagnostics, Richmond Hill, Canada). As part of the present study protocol, these isolates were retrieved and identification tests were repeated as well as on bacteria identified as non-fermentative Gram-negative rods other than *B. pseudomallei*. For all *B. pseudomallei* isolates, minimal inhibitory

concentration (MIC) values were determined by means of the Etest (Biodisk, Solna, Sweden) on Mueller Hinton agar (Hercules, California, U.S.) for the following antibiotics: doxycycline, amoxicillin-clavulanic acid, chloramphenicol, SMX-TMP, ceftazidime and meropenem. Interpretive criteria were those defined by the Clinical and Laboratory Standards Institute for *B. pseudomallei* [12], except for chloramphenicol, for which susceptibility and resistance breakpoints defined for *Enterobacteriaceae* were used (≤ 8 mg/L and ≥32 mg/L respectively). For assessment of antimicrobial resistance rates, only the first isolate was considered unless the subsequent isolate was recovered more than one month after the first one, *i.e.* after completing the initial attack phase of therapy.

Clinical and demographic data

From the microbiologically proven cases, demographic and clinical data were registered based on the patients' charts. Risk factors as described by Suputtamongkol and coworkers [13] were noted if explicitly recorded in the patients' charts. The rainy season was defined as the period between May 15th and November 15th. Clinical presentations were defined as follows: 'fever' was defined as the measurement of a body temperature of ≥37.5°C and/or a notion of fever from the patients' history. 'Shock' was defined as presenting with a mean arterial pressure (MAP) of < 60 mmHg despite adequate intravenous fluid resuscitation [10]. 'Multiple organ failure' (MOF) was defined as the clinical or biochemical failure of at least two end-organs [10, 14]. 'Pneumonia' was noted according to the presence of clinical and/or radiographic signs. 'Recurrent melioidosis' was defined as the development of new symptoms and signs of infection in association with a positive culture for *B. pseudomallei* after an initial response to therapy [15, 16]. Empirical treatment was considered 'appropriate' if it contained either ceftazidime, a carbapenem antibiotic or amoxicillin-clavulanic acid with or without SMX-TMP.

Statistical analysis

Risk factors were assessed by univariate analysis: Fisher's exact test was used for categorical variables, and Student's t-test or appropriate non-parametric tests for continuous variables. Associations were considered statistically significant at p-values < 0.05. Data were analyzed using Stata version 10.2 (Stata Corp, College Station, Texas, USA) and Excel 2003 (Microsoft Corporation, Redmond, Washington, USA).

Ethical approval

Ethical approval was granted from the review boards at the Institute of Tropical Medicine, Antwerp, the University Hospital Antwerp and the National Ethical Committee in Phnom Penh, Cambodia.

Results

Demographics and epidemiology

Melioidosis was diagnosed in 58 patients with a mean age of 49 years (range 18-73); 34 of them (58.6%) were male. The infections' seasonal pattern and the geographic distribution of the patients' homes (n = 56) are shown in Figures 1 and 2 respectively. The majority of patients (39/58; 67.3 %) were diagnosed during or shortly after the rainy season. Patients originated from 14 provinces, mainly the southeastern lowlands.

From 22 patients, professional occupation was recorded. The majority (18/22, 81.8%) were farmers; in addition, there was a policeman, a soldier, a construction worker and a motorcycle taxi driver.

In two-thirds of patients (n = 39, 67.2%) *B. pseudomallei* was recovered from a BSI, representing 12.0% of all clinical significant organisms (n = 328) recovered from BSI's during the study period, and 1.0% of all 3,976 SIRS episodes.

Risk factors for acquisition of melioidosis

Information on risk factors was available for 51 patients. Risk factors included diabetes mellitus (n = 34, 58.6%), alcoholism (n = 7, 12.1%) and corticosteroid use (n = 3, 5.2%), systemic lupus erythematodes (SLE, n = 2), chronic renal failure (n = 2), and chronic hepatitis, heart failure and tuberculosis (n = 1 each). In six patients, more than one risk factor was noted whereas in three patients no apparent risk factor was recorded. Information on serology for the human immunodeficiency virus (HIV) was retrieved in 20 files, all were negative. No known HIV-positive patient was admitted with melioidosis.

Clinical presentation

Fever was the most common presenting symptom (42/58; 72.4%). In addition, patients presented with cough (n = 18, 31.0%), dyspnoea (n = 13; 22.4%), weight loss > 10% (n = 10, 17.2%), abdominal pain (n = 10; 17.2%) or altered mental status (n = 6). Seventeen patients (29.3%) presented with shock and/or MOF. The median duration of symptoms to attendance was 28 days (range 1-730). Six patients had symptoms for about two months. Another six patients had very longstanding histories of three (n = 3), eight, nine and 24 months (n = 1 each) respectively. Nineteen patients (32.8%) had visited local or other health care services for their symptoms prior to attendance at SHCH.

The majority of patients (39/58, 67.2 %) presented with BSI with or without pneumonia (Table 1). During the time of the study period and along the introduction of pus and wound cultures, *B. pseudomallei* isolates were increasingly recovered from non-blood specimens, representing four out

of 27 (14.8%) isolates in 2008 versus 13 out of 24 (54.2 %) in 2009. Involvement of the lungs was noted in 28 (48.3%) cases. Besides, patients presented with infection in the skin and soft tissue (SSTI, n = 17), in bone and joints (n = 8), as urogenital infections (n = 4) and as deep organ abscesses in the spleen (n = 8), the liver (n = 5), the psoas muscle and the thyroid gland (n= 1 each). As shown in Table 1, the infection was often multifocal.

Laboratory values

The mean value of hemoglobin was 10.7 g/dL (range 5.5-18.0)), the mean white blood cell count 12.0 10^9/L (range 2.2-29.3 10^9) and mean platelets' count 261.8 10^9/L (range 34.0-1022.0 10^9). Mean blood sugar was 269.4 mg/dL (range 48.6-496.8), median serum aspartate aminotransferase (sAST) and serum alanine aminotransferase (sALT) were 70 U/L (interquartile range (IQR) 43.0-118.0) and 67 U/L (IQR 35.0-105.0) respectively.

Radiological presentation

Data of the medical imaging were available from 19 chest radiograph and 14 abdominal ultrasound examinations. Pulmonary involvement was mainly described as lobar infiltrates in the upper or lower lobes of either lungs (n = 7) or as a bilateral micro nodular ('metastasis-like') pattern (n = 5). Pleural effusions were noted in seven patients; in four this was the only presentation, in three it accompanied pulmonary infiltrates. Other abnormalities included a pulmonary abscess, a coin lesion in the lingula and enlarged mediastinal lymph nodes with scar tissue in both upper lobes (n = 1 for each). On abdominal ultrasound examination, deep abscesses were seen in liver, spleen, psoas and the thyroid gland and were mainly described as multiloculated (pseudotumoral) hypoechogenic lesions.

Treatment and outcome

Overall, 30 out of 58 (51.7%) patients died, 25 (43.1%) survived; for three patients, no outcome data were available. Among the survivors, 22 (88.0%) patients recovered without recurrence, the remaining three were lost to follow up in the course of the maintenance treatment phase. The mean duration of follow-up at the time of writing was 12.8 months (range 3.5-28). Three patients presented with recurrent disease; two of them survived without further events (Box 1).

Mortality occurred early in the admission: 19 (63.3%) non-survivors died within the first week. Among them, seven were referred to another hospital because no bed was available in SHCH, three patients died at home before the culture results were known, one was taken home for palliative care after 18 days of admission. Table 2 displays the univariate analysis of risk factors for mortality.

Significant risk factors were presentation with BSI (RR 6.8 (1.82-25.5), p < 0.001), shock/MOF (4.59 (1.60-13.32), p < 0.001) or not receiving appropriate empirical therapy (RR 3.5 (2.07-5.90), p < 0.001). In contrast, those presenting with skin and soft tissue infections (SSTI) had more favorable outcomes than those with other presentations (0.48 (0.24-0.97), p = 0.023). Platelet counts were significantly lower in those who died (189.6 10^9/L) compared to those who survived (355.0 10^9/L, p<0.001).

Details on treatment given were available for 53 patients. Eighteen (34.0 %) received inappropriate empirical therapy; all of them died early or were discharged on their families' request for palliative home care. Thirty-five patients had received appropriate treatment, either from admission or early during the course of the hospitalization: 23 patients were given ceftazidime (2 g q8 for at least 14 days) with or without SMX-TMP (30 mg/kg q12), six received amoxicillin-clavulanic acid (875-1000 mg q8) with or without SMX-TMP and another six SMX-TMP with doxycycline (200 mg q24). Twenty-three patients continued on maintenance therapy: mostly SMX-TMP with or without doxycycline (n = 22), one patient received amoxicillin-clavulanic acid with SMX-TMP. Total treatment duration ranged from three to six months.

In one patient a late treatment failure was noted: a 43-year old soldier with chronic hepatitis and diabetes presented with fever, pneumonia and multiple spleen abscesses; *B. pseudomallei* was isolated from blood cultures. He was treated with ceftazidime + SMX-TMP for two weeks followed by SMX-TMP + doxycyclin. In the fourth month of his maintenance therapy, he died of a sudden rupture of the spleen abscess.

Microbiological data

During the study period, seventy-one isolates of *B. pseudomallei* were recovered from 58 patients (Table 3). Nearly 60% (41/72) of them were isolated from blood; one quarter (18/71) was recovered from wound or abscess fluid. All available isolates initially identified as *B. pseudomallei* were confirmed upon retesting. One additional isolate was added out of 34 isolates originally identified as other Gram-negative non-fermentative species (Box 1, patient 2). In two patients, successive isolates were recovered more than one month after the first one: in one patient, *B. pseudomallei* was recovered from an abscess 137 days after the first isolate (Box 1, patient1). In the other patient, the second isolate was recovered from blood, 231 days after the first (Box 1 patient 2).

In total, 54 isolates (52 first and 2 successive isolates) were used for assessment of resistance rates.

For the blood culture isolates (n = 41), median time-to-positivity was four days for the manual system (n = 31) and three days for the BacTalert system (n = 11), with a range of 2-8 days for both methods.

Table 4 lists the MIC-distribution for the different antibiotics with known activity against melioidosis. No resistance was noted for ceftazidime, meropenem, amoxicillin-clavulanic acid, SMX-TMP or doxycycline, but 12 (22.2 %) isolates had MIC-values equal to the susceptibility breakpoint susceptibility for chloramphenicol. Of note, three isolates showed ceftazidime MIC values of 3 µg/mL, which is close to the susceptibility breakpoint. All three isolates equally showed MIC-values above the MIC90 for amoxicillin-clavulanic acid and meropenem. Two of these isolates were recovered as first and successive isolate from one patient.

Discussion

This study presents the clinical picture and microbiological data of melioidosis as observed in 58 adult patients from across Cambodia. Our findings complement the recently published data on melioidosis in children from Cambodia [9]. Patient were predominantly male and suffering of diabetes mellitus and presented mostly during the rainy season. We observed a broad range of clinical presentations, predominantly pneumonia with or without BSI, deep organ abscesses and SSTI. Mortality was high (> 50%), especially for patients presenting with BSI, sepsis or MOF, and for those who did not receive appropriate empirical therapy. Besides the intrinsic resistance to the commonly used antibiotics, we did not observe acquired resistance for the antibiotics of choice.

There were several limitations in our study. First, the retrospective nature of the present study did not allow tracing into detail clinical data, risk factors (such as exposure to soil and water), outcome and factors related to recurrence of disease. Neither did it allow the calculation of population-based incidence data. In addition, SHCH offers free treatment to the poor and attracts patients across the country. This may have caused a selection bias to very sick patients with prolonged disease courses leading to an underrepresentation of acute lethal forms and minor clinical illness. Finally, at present we did not yet investigate the isolates to the genetic level, precluding study of evolutionary relationships and distinction between re-infections and relapses. However, the phenotypic characteristics we used to identify *B. pseudomallei* isolates have been validated against molecular reference standards and are considered as accurate tools for species identification [11].

In general, the risk factors and epidemiology in this study were similar to those observed in northeastern Thailand [1, 13]. As in these settings, the majority of our patients were older adults, with a male predominance. The observation has been explained by a higher degree of exposure in males [17] or a higher male susceptibility for sepsis in general. Diabetes mellitus was the most important risk factor (58.6 %); this in line with the 23-60% presence of diabetes among patients with melioidosis in Thailand, 37% in Australia and 38-57% in Malaysia and Singapore [1, 18-20]. Diabetes is quickly emerging in Cambodia, and remains a difficult-to-treat chronic disease in rural poor settings [21, 22]. In view of the demographic shifts towards an older and more diabetes-prone population, melioidosis may be emerging as well.

We saw a predominance of patients coming from the southern and southeastern provinces (*e.g.* Kampong Speu, Prey Veng, Kandal, Kampong Cham). These are among the country's lowest areas, located in the alluvial plain of the Mekong River, with less than four months of dry season per year and intensive rice farming activities [23]. More information from clinical cases presenting in provincial hospitals and from environmental sampling is needed to clarify on Cambodia's 'melioidosis hot spots'.

Most patients were seen during or shortly after the wet season, in line with observations from neighboring countries, *e.g.* Thailand [13], and Australia [24]. In 2009, we observed a high incidence of cases which extended beyond the expected end of the rainy season until the end of the year. This correlated with a more intense and prolonged rainy season in the entire Southeast Asian region that year. The reason for the apparent link between rainfall and melioidosis incidence is still subject to debate: an increased bacterial load in the environment and a seasonally driven increased exposure (*i.e.* farming activities) have been suggested [25].

Melioidosis presented among our patients as an important cause of severe community-acquired BSI, accounting for 12.0 % of all with BSI attending the hospital, which is in line with earlier Thai data (*i.e.* 18% of community-acquired BSI) [1]. Similarities with Thai and Australian data were also observed in the main clinical presentations *e.g.* BSI (67.2% in the present study compared to 60% in Thailand [26] and community-acquired pneumonia (CAP) (48.3% compared to 46% in Australia [27]. Soft tissue and deep organ abscesses were also frequent; especially the finding of a spleen abscess in a melioidosis-endemic area should trigger the diagnosis of melioidosis as the list of alternative diagnoses is rather short with *B. pseudomallei* being by far the most frequent cause [28].

In our patients, it was not possible to distinguish primo-infection or re-infection from relapse, but the strong seasonal link suggests many were recent infections. In contrast, most of the above

mentioned 'recurrent' cases were probably relapses due to insufficient treatment of the first episode as described in Thai and Australian patients [15, 29]. This emphasizes the need for an intense follow-up during and after the complete treatment course.

We noted a high mortality, especially among the patients with BSI and/or pneumonia, in those presenting with shock or MOF, and/or with inappropriate empirical therapy. High mortality in the most vulnerable patient groups has also been described in more affluent settings, *e.g.* 72 % mortality among pneumonic patients in Singapore [30]; 90% and 86% in Thai and Australian patients with BSI respectively [27, 31, 32].

Potential interventions to decrease mortality due to melioidosis include improved sepsis care and ensured availability of effective drugs such as ceftazidime, carbapenems, and/or amoxicillin-clavulanic acid. The latter two have recently been included in SHCH's essential drug list: ceftazidime is the drug of choice for the initial treatment of severe melioidosis, whereas amoxicillin-clavulanic acid is positioned for milder cases of melioidosis and for the empirical treatment of serious CAP.

Timely diagnosis may decrease the delay before administration of appropriate antibiotic treatment; in that way, the presently observed time-to-detection of blood cultures is of concern. The long detection delays are striking, especially since *B. pseudomallei* tends to cause heavy bacterial loads in the blood [33]. Continuous monitoring of BacTalert blood culture bottles in the dedicated automate probably will reduce the time-to-detection. An alternative may be to perform blind subcultures after 48 hours of incubation [34]. The identification scheme implemented in the microbiology laboratory proved to be reliable, as illustrated by the fact that only one out of 34 isolates that had initially been identified during routine work-up as a non-fermentative Gram-negative rod other than *B. pseudomallei* was identified as *B. pseudomallei* upon repeat identification .

The antibiotic susceptibility patterns of the present isolates were in line with the findings in other series [17]. Although we presently did not demonstrate resistance to any of the key antibiotics used in the attack and the maintenance phases, it should be noted that resistance is rare but can occur during therapy, reason for the need of follow-up blood cultures during treatment [35].

During the 19-month study period, we observed a learning curve on melioidosis at several levels in the hospital. Even though melioidosis has been well known for several years in the southeast Asian region [2], the concept of 'melioidosis' was unfamiliar to most clinicians and other health care workers. The described experience incited patient discussions, journal clubs and trainings about melioidosis and allowed us to design locally adapted treatment guidelines for sepsis and CAP.

Our findings may have also an impact at the national level, especially when it comes to early detection and treatment. Awareness has to be raised among first line health care workers and high-risk patients groups (*e.g.* diabetes patients) [30, 36] about the various clinical presentations and therapeutic measures of melioidosis. The development of quality-assured and affordable microbiological capacity throughout the country is equally important in the broader picture of surveillance and containment of antimicrobial resistance. The need for more widespread availability of expensive broad spectrum antibiotics with proven clinical efficacy in melioidosis (such as ceftazidime and injectable amoxicillin-clavulanic acid) is clear, but incautious implementation might have an important impact on the country's and patients' drug expenses and on local antimicrobial resistance patterns. Careful adaption of the existing local treatment guidelines is essential and has been successful in other settings *e.g.* northern Australia [37].

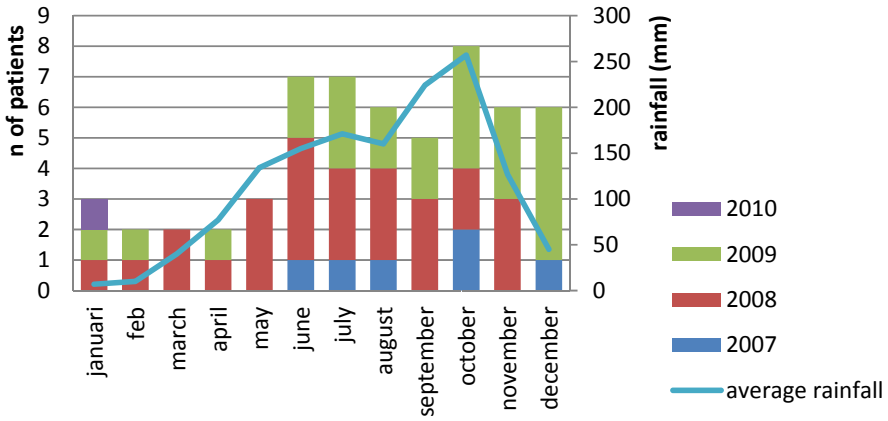
Further research on melioidosis in Cambodia and in other low resources tropical settings is highly needed. More accurate assessments of its prevalence and impact at population level, among diabetes and CAP patients will be necessary as well as the improvement of fast, reliable and affordable diagnostics and feasible ways to improve patients' outcomes. In addition, the impact of these findings on antibiotic use patterns country wide will need to be closely followed.

Conclusion

Melioidosis is a common cause of severe community-acquired BSI and pneumonia in adults across Cambodia affecting diabetic patients in particular. The mortality is high, and related to clinical presentation and adequate empirical treatment. This may have important repercussions on national and local diagnostic and treatment guidelines.

Figures and tables

Figure 1. Monthly number of patients with melioidosis in SHCH (Cambodia), 2007-2010.



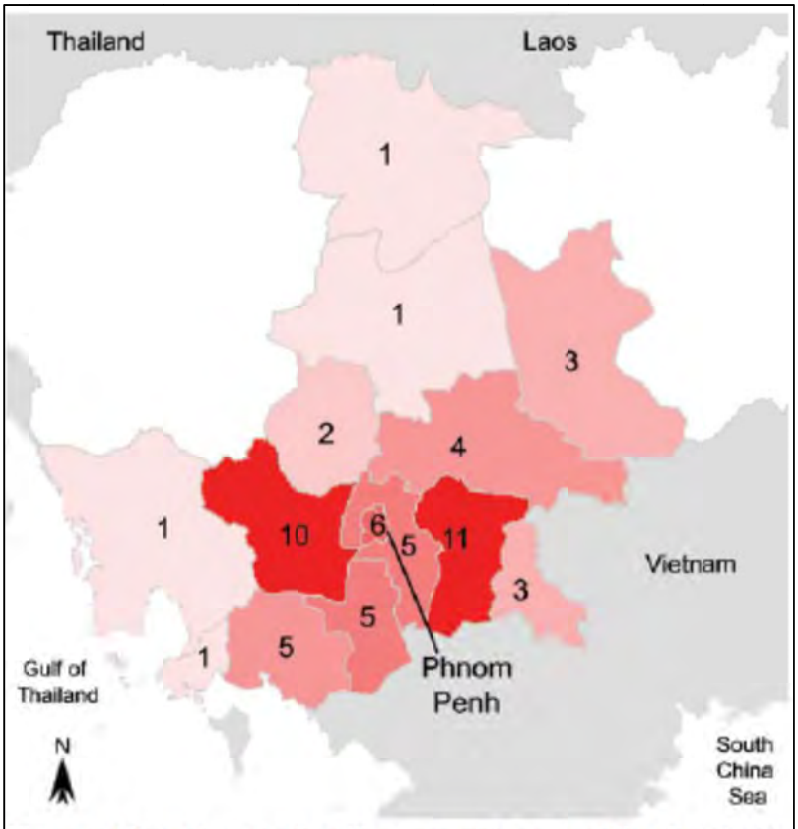


Figure 2. Map of Cambodia with geographic origin of the 58 patients with melioidosis diagnosed during July 1, 2007–January 31, 2010.

Table 1. Focus of infection in 58 patients with melioidosis.

	Bloodstream infection	
	Yes	No*
pneumonia only	11	-
pneumonia + deep organ abscess	5	1
pneumonia + SSTI	1	2
pneumonia+ deep organ abscess + SSTI	1	1
pneumonia+ bone/joint infection	2	1
pneumonia + bone/joint infection + SSTI	2	-
pneumonia + urogenital infection + SSTI	-	1
deep organ abscess only	2	-
deep organ abscess + SSTI	1	2
deep organ abscess + bone/joint infection + SSTI	-	1
deep organ abscess + urogenital infection	-	1
SSTI only	1	7
bone/joint infection only	2	-
urogenital infection only	1	1
no apparent focus/disseminated	10	1
Total	39	19

* *B. pseudomallei* not isolated from blood cultures or no blood cultures taken , SSTI: skin and soft tissue infection

Table 2. Predictors of mortality in 58 patients with melioidosis*

		Total	Died	Relative risk (95% CI)	p-value
Risk factors					
Age >55 y	yes	24	14	1.13 (0.70-1.83)	0.786
	no	31	16		
Male gender	yes	31	18	1.16 (0.70-1.91)	0.595
	n o	24	12		
Rainy season	yes	36	23	1.37 (0.76-2.48)	0.351
	no	19	7		
Diabetes mellitus	yes	32	14	0.70 (0.41-1.21)	0.359
	no	16	10		
Alcoholism	yes	7	6	1.96 (1.19-3.22)	0.092
	no	32	14		
Clinical presentation					
Duration of illness < 2 months	yes	12	3	0.44 (0.16-1.26)	0.152
	no	23	13		
Bloodstream infection	yes	37	28	**6.81 (1.82-25.50)**	**<0.001**
	no	18	2		
Pneumonia	yes	28	18	1.52 (0.90-2.57)	0.172
	no	26	11		
Deep abscesses	yes	15	6	0.80 (0.38-1.67)	0.742
	no	24	12		
Bone/joint infection	yes	8	4	1.04 (0.47-2.28)	1.000
	no	29	14		
Urogenital infection	yes	5	1	0.38 (0.064-2.25)	0.345
	no	38	20		
SSTI	yes	19	6	**0.48 (0.24-0.97)**	**0.023**
	no	35	23		
Shock and/or MOF	yes	17	13	**4.59 (1.60-13.32)**	**< 0.001**
	no	18	3		
Therapy					
Inappropriate empirical therapy	yes	18	18	**3.5 (2.07-5.90)**	**< 0.001**
	no	35	10		

* for some of the 58 patients not all information on outcome predictors was available; MOF: multiple organ failure, SSTI: skin and soft tissue infection

Table 3. Specimen type from which *Burkholderia pseudomallei* isolates were recovered, for 58 patients.

Origin	1st isolate	2nd isolate	3d isolate	Total
Blood	38 (35)*	3 (1)	0	41 (36)
Wound /Abscess	14 (11)	3 (1)	1	18 (12)
Sputum	1 (1)	1	0	2 (1)
Urine	3 (3)	3	0	6 (3)
Bone marrow	0	1	1	2
Joint fluid	2 (2)	0	0	2 (2)
Total	58 (52)	11 (2)	2	71 (54)

**n isolates available for further susceptibility analysis (see Table 4)*

Table 4. MIC-values (µg/mL) distribution for 54 *B. pseudomallei* isolates as deteremined by E-test. Data refer to numbers of isolates.

	MIC (µg/mL)										MIC50	MIC90	breakpoints (µg/mL)*	
	0.38	0.5	0.75	1	1.5	2	3	4	6	8			S	R
meropenem	3	29	16	1	3	2	-	-	-	-	0.5	1	≤ 4	≥ 16
doxycycline**	-	13	19	18	3	1	-	-	-	-	0.75	1	≤ 4	≥ 16
ceftazidime**	-	2	0	17	25	7	2	-	-	-	1.5	2	≤ 8	≥ 32
amoxicillin-clavulanic acid	-	1	0	13	30	7	2	1	-	-	1.5	2	≤ 8	≥ 32
chloramphenicol	-	-	-	-	1	1	0	17	16	12	6	8	≤ 8	≥ 32

	MIC (µg/ml)															
	0.032	0.032	0.047	0.064	0.094	0.125	0.19	0.25	0.38	0.75	1	1.5	3			
sulfamethoxazole-trimethoprim	3	1	7	11	5	9	4	7	1	1	3	1	1	0.125	0.75	≤ 2	≥ 4

**Breakpoints: S = susceptible, R = resistant*

*** 53 isolates included*

Box 1. Patients with recurrent melioidosis in SHCH, 2007-2010.

Patient 1: A 55 year old policeman was admitted with a 6-months history of intermittent fever, abscesses of the right scrotum and spermatic cord, from which *B. pseudomallei* was isolated. Revision of the medical file revealed that he had attended 6 months earlier with a spleen abscess and growth of *B. pseudomallei* from urine. He had then partially recovered on a short course of ceftriaxone and amoxicillin-clavulanic acid but remained with intermittent fever until re-admission. He finally recovered after a 2 weeks course of ceftazidime + SMX-TMP followed by 6 months of SMX-TMP + doxycyclin and remained without relapse for 14 months now.

Patient 2: A 72 year old diabetic man presented with fever, pneumonia, stupor and MOF; he was treated empirically with ceftriaxone, metronidazole and amikacin and died later at home. Blood cultures yielded *B. pseudomallei* after 3 days. File revision showed he was treated 7 months earlier for a bloodstream infection due to '*Pseudomonas aeruginosa*'. Revision of the stored isolate led to a post hoc correction of the identification into *B. pseudomallei*.

Patient 3: A 62 year old diabetic man was admitted with fever and chronic cough. He was diagnosed with a pneumonia and pleural effusion at the left thorax base and multiple spleen abscesses. Blood cultures grew *B. pseudomallei* after 3 days. Two years earlier, he had been diagnosed with a buttock abscess and bloodstream infection with (not further identified) Gram-negative bacteria. He had been treated with amoxicillin-clavulanic acid for 2 weeks but remained with a chronic cough for 2 years until the re- admission. He fully recovered after a treatment course with ceftazidime + SMX-TMP for 2 weeks followed by SMX-TMP + doxycyclin for 6 months and remained without relapse for 28 months now.

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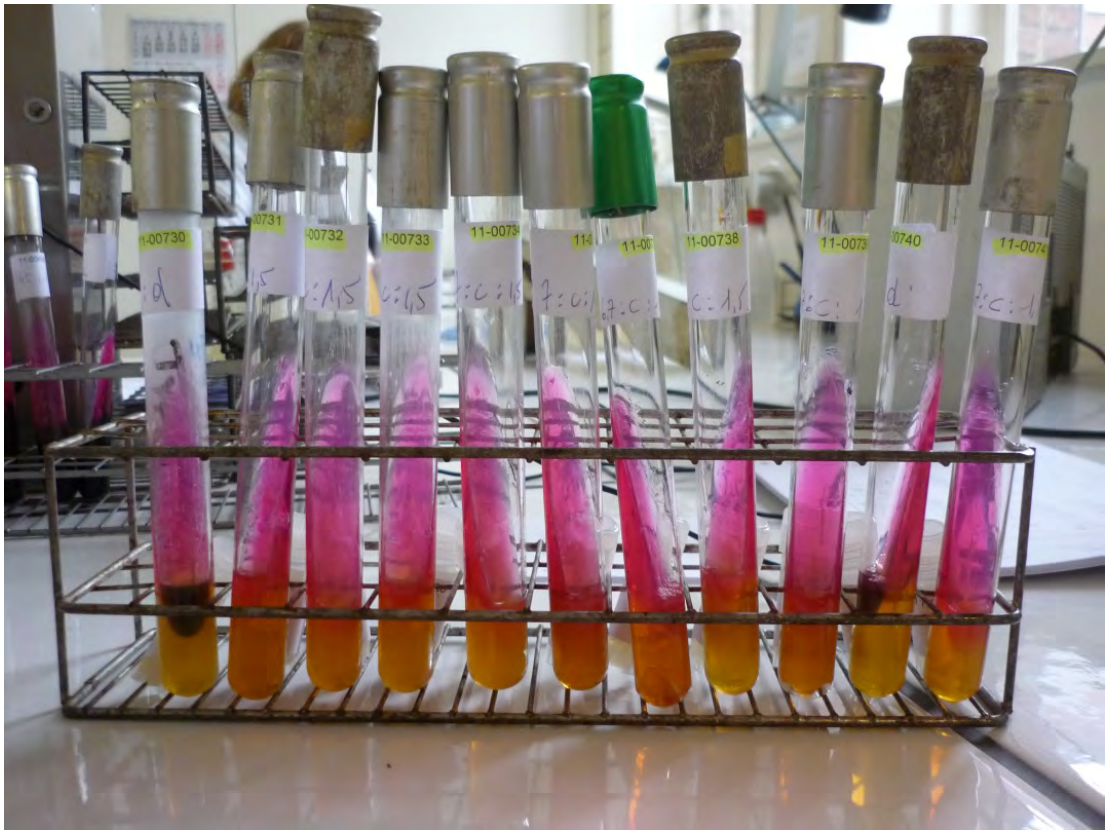
Chapter 4
The classical tropical bloodstream infection: *Salmonella enterica*.



Culture of *Salmonella* spp. in a Kligler's medium revealed that we isolated a high number of *Salmonella* Choleraesuis (pink-yellow discoloration), a non-typhoid *Salmonella* serovar which is transmitted through contact with infected pigs. Azithromycin is considered a 'salvage' antibiotic for *Salmonella* treatment, yet we found 70% of these *Salmonella* Choleraesuis to be azithromycin resistant.

Azithromycin and ciprofloxacin resistance in *Salmonella* bloodstream infections in Cambodian adults.

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Abstract

Background

Salmonella enterica is a frequent cause of bloodstream infection (BSI) in Asia but few data are available from Cambodia. We describe *Salmonella* BSI isolates recovered from patients presenting at Sihanouk Hospital Centre of Hope, Phnom Penh, Cambodia (July 2007-December 2010).

Material and methods

Blood was cultured as part of a microbiological surveillance study. Identification of *Salmonella* isolates was performed by conventional methods and serotyping. Antibiotic susceptibilities were assessed using disk diffusion, MicroScan® and Etest macromethod. Clonal relationships were assessed by Pulsed Field Gel Electrophoresis; PCR and sequencing for detection of mutations in Gyrase and Topoisomerase IV and presence of qnr genes.

Results

Seventy-two *Salmonella* isolates grew from 58 patients (mean age 34.2 years, range 8-71). Twenty isolates were identified as *Salmonella* Typhi, 2 as *Salmonella* Paratyphi A, 37 as *Salmonella* Choleraesuis and 13 as other non-typhoid *Salmonella* spp. Infection with human immunodeficiency virus (HIV) was present in 21 of 24 (87.5%) patients with *Salmonella* Choleraesuis BSI. Five patients (8.7%) had at least one recurrent infection, all with *Salmonella* Choleraesuis; five patients died. Overall, multi drug resistance (*i.e.* co-resistance to ampicillin, sulphamethoxazole-trimethoprim and chloramphenicol) was high (42/59 isolates, 71.2%). *Salmonella* Typhi displayed high rates of decreased ciprofloxacin susceptibility (18/20 isolates, 90.0%), while azithromycin resistance was very common in *Salmonella* Choleraesuis (17/24 isolates, 70.8%). Two *Salmonella* Choleraesuis isolates were extended spectrum beta-lactamase producer.

Conclusion

Resistance rates in *Salmonella* spp. in Cambodia are alarming, in particular for azithromycin and ciprofloxacin. This warrants further surveillance and revision of treatment guidelines.

Introduction

Salmonella enterica is an important cause of morbidity and mortality worldwide[1, 2]. *Salmonella enterica* serovar Typhi is the etiologic agent of typhoid fever while non-typhoid *Salmonella* spp. (NTS) are associated with gastroenteritis and invasive infections in children, the elderly and immune depressed [3]. Both *Salmonella* Typhi and NTS are among the most frequent pathogens causing bloodstream infections (BSI) in tropical low-resource settings[4]. The highest incidence of *Salmonella* infections worldwide occurs in Asia[1, 5], mainly in South- and Southeast Asia, where isolates show high rates of antibiotic resistance[6, 7]. Although fluoroquinolones are drugs of choice to treat invasive *Salmonella* infections, decreased susceptibility to ciprofloxacin (DCS) is increasing quickly worldwide[2]. Azithromycin and ceftriaxone have been recommended as treatment alternatives for typhoid fever in case of DCS[8-10].

Little is known about the epidemiology and the extent of antibiotic resistance in invasive human *Salmonella* infections in Cambodia. As part of a microbiological surveillance study on the causes of BSI in Cambodian adults and an antibiotic stewardship program, we aimed to assess the antibiotic resistance patterns of invasive salmonellosis in this population.

Methods

Study setting and period

Sihanouk Hospital Centre of HOPE (SHCH) is a 40-bed non-government referral hospital in Phnom Penh, Cambodia. Microbiological services were installed in 2005. From July 2007 until June 2011 a prospective BSI surveillance program was carried out.

Patients and blood culture sampling

From all adult patients presenting with signs of the Systemic Inflammatory Response Syndrome (SIRS)[11], blood cultures were taken taken as described in Chapter 2.

Microbiological work-up of isolates

Isolates identified as *Salmonella* spp. at SHCH were retrieved from -70°C, checked for purity and further worked up at the Institute of Tropical Medicine (Antwerp, Belgium) and the Scientific Institute of Public Health (Brussels, Belgium). Serotyping was carried out by slide agglutination with commercial antisera according to the Kauffmann-White scheme[12].

Clonal relationships were assessed by pulsed field gel electrophoresis (PFGE) according to the PulseNet Europe protocol[13]. Genomic DNA was digested with XbaI restriction enzymes (New-England

Biolab, Leusden, Netherlands), *S.* Braenderup H9812 was used as a size marker. Profiles were analyzed using the Dice coefficient[14] and the unweighted-pair group method using average linkages, with a tolerance of 1%.

For the compilation of the resistance data, only the first isolate per BSI episode (defined as a 14-day period following the first day of BSI diagnosis) was considered. Recurrent infections were defined as a new BSI episode with an identical *Salmonella* serovar at least 14 days after the former isolate and after appropriate treatment of the patient. Recurrent isolates were considered as duplicate isolates and not compiled into the resistance overview; their resistance data were considered separately.

Antibiotic susceptibilities were assessed by disk diffusion (using Neo-Sensitabs™, Rosco Diagnostica, Taastrup, Denmark) and MicroScan (Combo 42, Siemens Healthcare Diagnostics, Deerfield, USA). Minimal inhibitory concentrations (MIC) for nalidixic acid (NA), ciprofloxacin, chloramphenicol and azithromycin were determined using the Etest macromethod (bioMérieux).
Breakpoints were those defined by the Clinical and Laboratory Standards Institute[15]; intermediately resistant isolates were considered as resistant. DCS was defined according to European Committee on Antimicrobial Susceptibility testing (EUCAST) guidelines, *i.e.* a MIC-value for ciprofloxacin > 0.064 µg/mL[16]. Multidrug resistance (MDR) was defined as co-resistance to the first line antibiotics ampicillin, chloramphenicol and sulphamethoxazole-trimethoprim (SMX-TMP). For azithromycin and *Enterobacteriaceae*, no breakpoints have been published. EUCAST mentions treatment of *Salmonella* Typhi infections with a MIC≤ 16 µg/mL and a recent publication proposed 16 µg/ml as 'epidemiological cutoff' value for wild type *Salmonella* spp.[17]. Detection and identification of ESBL producing bla genes was performed by a commercial multiplex ligation PCR microarray CT 101 (Check-Points Health BV, Wageningen, The Netherlands)[18].

Screening for mutations in the quinolone resistance-determining region (QRDR) was performed by amplification of a fragment of the *gyr*A, *gyr*B, and *par*C genes containing the QRDR as previously described[19] and sequencing of the fragments on a CEQ 2000 DNA sequencer (Beckman Coulter, High Wycombe, United Kingdom), using the DTSC-2 method. The sequences were compared and analyzed by Genestream software (Institut de Génétique Humaine, Montpellier, France). The presence of the plasmid-mediated quinolone resistance qnr genes (qnrA, qnrB, and qnrS) was determined using PCR[20]

Ethical considerations

Ethical approval was granted from the Institute of Tropical Medicine, Antwerp, the University

Hospital Antwerp and the National Ethical Committee, Phnom Penh, Cambodia on 9/6/2008 and 12/01/2009 respectively. Blood cultures were sampled as part of routine clinical care, not requiring prior informed patient consent. Patients were identified with a unique hospital number. For the clinical and epidemiological data, no other data besides those noted in the routine medical files were used.

Results

Demographic and clinical data

From 6881 blood cultures drawn during the study period, 72 non-duplicate *Salmonella enterica* isolates were recovered from 58 adult patients, representing 11.5 % of all clinically significant organisms (CSO). These isolates were recovered from 59 first BSI episodes and 13 recurrent episodes (Figure 1). The serovars included *Salmonella* Choleraesuis (n = 37; 51.4%) and *Salmonella* Typhi (n = 20; 27.8%) followed by *Salmonella* Enteritidis (n = 7; 9.7%), *Salmonella* Typhimurium (n = 4; 5.6%), *Salmonella* Paratyphi A (n = 2; 2.8%), *Salmonella* London and *Salmonella* Amsterdam (n = 1; 1.4% each).

The mean age of patients with *Salmonella* BSI was 34.2 years (range 8-71), 51.7% were women. They came from at least 10 different provinces, mainly the greater Phnom Penh area (n = 11; 19.0%) and Kandal province (n = 7; 12.1%). The majority of *Salmonella* BSI occurred during the rainy months April to November (n = 57; 79.1%); no apparent other temporal or geographical clustering was noted.

Co-morbidity was present in 36 (62.1%) patients, mainly human immunodeficiency virus (HIV) infection (n = 32; 55.2%); we also noted systemic lupus erythematodes (n = 2; 3.4%), thalassemia and valvular heart disease (one patient each). For 13 HIV-infected patients, *Salmonella* BSI was the indicator disease for HIV-infection; only three HIV-patients were on antiretroviral treatment at the time of the BSI. The median CD4-cell count was 22 per microliter (range 2-253), concurrent opportunistic infections (OI) included tuberculosis (n = 6) and cryptococcal meningitis (n = 3). Of note, *Salmonella* Choleraesuis was the most common pathogen in HIV-infected patients (21/32, 65.6%) whereas *Salmonella* Typhi was predominantly recovered from HIV-negative patients (19/26, 73.1%). Of the 24 patients with *Salmonella* Choleraesuis BSI, 12 (50.0%) presented with fever, six (25.0%) with abdominal pain and diarrhea and five (20.8%) with dyspnea and dry cough.

Patients were treated empirically with either ceftriaxone, amoxicillin-clavulanic acid or ciprofloxacin (or subsequent administration of these antibiotics) for a mean duration of 11.7 days (range 1-21). Additional treatment for HIV-related OI included SMX-TMP, fluconazole and tuberculostatic drugs.
A total of five patients infected with *Salmonella* spp.(8.6%) had one or more recurrent BSI episodes with the same serovar, all *Salmonella* Choleraesuis (Figure 1). The mean interval to recurrence was 4.5 weeks (range 2-10 weeks). One HIV-patient had a *Salmonella* Typhimurium BSI eight months after being treated for *Salmonella* Enteritidis BSI. Five patients (8.6%) died. Four of them had been infected by *Salmonella* Choleraesuis and one by *Salmonella* Typhimurium; all were HIV-infected. The median duration between the diagnosis of *Salmonella* BSI and death was 24 days (range 13-61 days).

Pulsed Field Gel Electrophoresis (PFGE)

For *Salmonella* Choleraesuis, three different PFGE profiles were obtained, of which Xb-Chol-1 was predominant (86.5%), including all 13 recurrent isolates (Figure 2). The PFGE profiles of 'first' and 'recurrent' isolates were identical per patient. No association between a particular PFGE profile and resistance profile was observed (data not shown).
All *Salmonella* Typhi isolates had a similar PFGE profile (*i.e.*Xb-Ty-1) whereas *Salmonella* Enteritidis and *Salmonella* Typhimurium presented with two and three different profiles respectively.

Antibiotic resistance

Antibiotic resistance data as assessed for the 59 'first' (*i.e.* non-recurrent) isolates are shown in Table 1. Of note, very high rates of MDR were seen in *Salmonella* Typhi (15/20 isolates, 75.0%) and *Salmonella* Choleraesuis (22/24 isolates, 91.7%) and to a lesser extent in other NTS (5/13 isolates, 38.5%).

DCS was particularly present among *Salmonella* Typhi isolates, with MIC50 and MIC90 of 0.25 µg/mL and 0.38 µg/mL respectively (Table 2). Thirty-one (88.6%) out of 35 DCS isolates displayed resistance to NA, with mutations in *gyr*A at either position 83 (n = 24) or 87 (n = 3) (Table 3). One *Salmonella* Typhimurium displayed full resistance to ciprofloxacin (MIC 6 µg/mL) confined to two mutations in gyrA (Ser83→Phe and Asp87→Asn) and one in parC (Ser80→Arg). Of note, four isolates (all NTS) displayed DCS but were NA susceptible: no mutations in *gyr*A or *par*C were observed; in two of them presence of *qnr*S1 was detected. In 22 of 24 *Salmonella* Choleraesuis and in all *Salmonella* Paratyphi A we detected a *par*C mutation in position 57, regardless of susceptibility patterns.
MIC-levels for azithromycin were particularly high in *Salmonella* Choleraesuis isolates, with MIC50 and MIC 90 as high as 32 and 128 µg/ml respectively (Table 4).

In the successive isolates from patients with recurrent *Salmonella* BSI, no differences in resistance patterns were noted, except in one *Salmonella* Choleraesuis (recovered 23 days after the first *Salmonella* Choleraesuis BSI episode), having acquired ESBL. Presence of ESBL was also detected in another patient with *Salmonella* Choleraesuis infection. Both ESBL-positive isolates carried bla_{CTX-M} genes, The former was confirmed as CTX-M group 9 and displayed also MDR and azithromycin resistance (MIC 32 µg/mL). In the latter (CTX-M group 1), we observed additional DCS (MIC 0.125 µg/mL)

Discussion

We described the serovar distribution and antibiotic susceptibility of 72 *Salmonella enterica* BSI isolates from Cambodian adults, and noted a predominance of *Salmonella* Typhi and *Salmonella* Choleraesuis. Besides MDR, *Salmonella* Typhi in particular displayed high rates of DCS, while *Salmonella* Choleraesuis was associated with advanced HIV-infection and remarkably high azithromycin resistance rates.

Our findings have several limitations. First, the study describes *Salmonella* BSI mainly in adults. As *Salmonella* spp. is an important pediatric pathogen in tropical low-resource settings[1, 3], data on its invasive infections in children are essential to complement the epidemiological picture of salmonellosis in Cambodia. Next, our clinical hospital data did not allow calculations of incidence and/or the true burden of disease because the population denominator and referral pattern were not known. In addition, the presence of an HIV-treatment center in the hospital may have led to a patient selection bias. In spite of these limitations our data shed new light on invasive *Salmonella* infections in Cambodia.

In HIV-negative patients, *Salmonella* Typhi was the most common serovar, with very high rates of MDR (75.0%) and DCS (90.0%). This confirms earlier trends from Cambodia as noted by Kasper and coworkers in 2009[21] describing 56% of MDR and 80% DCS in *Salmonella* Typhi. The presence of MDR and DCS has been observed in other Asian countries, albeit with important differences. A survey on typhoid fever in five countries[22] revealed MDR rates as variable as 65% in Pakistan, 22% in Vietnam, 7% in India and 0% in China/Indonesia whereas rates of NA resistant *Salmonella* Typhi (NARST) ranged similarly between 57-59% (India, Pakistan), 44% (Vietnam) and 0% (China, Indonesia). Since the early 1990's, southern Vietnam has been particularly mentioned as a regional 'typhoid resistance hotspot' with NARST/DCS rates as high as 90-98%[10, 23]. The geographical location of Cambodia in the vicinity of this regional 'hotspot' may be one of the explanations for the high rates of DCS among our

patients with typhoid fever, given the intense cross-border traffic between the two countries. In addition, the uncontrolled use of ciprofloxacin and other antibiotics and the limited access to safe water and sanitation services[24] probably add to selection and spread of MDR and DCS isolates.

In Vietnam, the Ser83→Phe substitution in *gyr*A was described as the predominant underlying resistance mechanism for DCS[23]. We observed this mutation also in all *Salmonella* Typhi isolates with combined DCS and NA resistance and to a lesser extent in *Salmonella* Choleraesuis and other NTS. According to the Cambodian National Treatment Guidelines[25] ciprofloxacin is the first choice treatment for presumed typhoid fever with ceftriaxone as alternative. Given the failure risk of a treatment course with ciprofloxacin for invasive salmonellosis with DCS as high as 36%[26], we think the empirical treatment of typhoid fever with ciprofloxacin should be abandoned in Cambodia. Alternatives could be azithromycin for uncomplicated cases and ceftriaxone for hospitalized patients. Gatifloxacin proved to be a safe, cheap and effective alternative treatment in Nepal[27] and Vietnam[28], but it is not widely distributed in Cambodia, and caution remains regarding its use in the elderly and in a setting with increasing rates of MDR tuberculosis.

In addition, these data and their subsequent therapeutic challenges urge the need for more and better yet affordable diagnostic microbiology in Cambodia. More and adequately working microbiology laboratories across the country are essential for the improvement of clinical care and for surveillance of bacterial resistance.

Among HIV-infected patients, *Salmonella* Choleraesuis was the most common serovar. It is a zoonotic pathogen causing paratyphoid in pigs and is an emerging cause of invasive infections in immune depressed patients in South-East and Eastern Asia[29]. The prevalence of *Salmonella* Choleraesuis was not yet described in Cambodia in swine nor in humans but it is a well-known pathogen in neighboring Thailand[30, 31].

All isolates in patients with recurrent *Salmonella* Choleraesuis BSI had PFGE profiles which were identical to the first isolate, which is suggestive for relapse rather than for reinfection although the small number of pulsotypes and the limited discriminatory power of PFGE using XbaI[32] should be taken into account. Given the context of advanced HIV-infection, relapse is the more likely interpretation[33]

Most *Salmonella* Choleraesuis isolates (70.8%) had azithromycin MIC-values exceeding 16 µg/mL. To our knowledge, this has not yet been described in a series of clinical *Salmonella* isolates from a single setting. Of note, also one *Salmonella* Typhi and *Salmonella* Enteritidis isolate displayed high

azithromycin MIC-values. This contrasts with the low azithromycin MIC data for *Salmonella* Typhi reported from Vietnam (MIC90 8-16 µg/mL[10, 34]), India and Egypt (MIC90 8 µg/mL [35, 36]). Azithromycin MIC-values up to 64 µg/mL in *Salmonella* Typhi and Paratyphi A from India were recently described[37], and a Finnish study revealed azithromycin MIC-values ≥32µg/mL in 1.9% of 1237 NTS isolates; half of them were isolated after travel to Thailand[38]. While considering the azithromycin resistance 'epidemiological cutoff' of 16 µg/mL[17], azithromycin resistance apparently presents an emerging problem as treatment failures have been described[39].

Possible mechanisms of azithromycin resistance include the presence of specific resistance genes (*e.g. mph*A, *mph*B, *erm*B), a mutation in *rlp*D or *rlp*V, or the acquisition of an efflux pump[40]. In Cambodia, generic azithromycin can be purchased over the counter of private clinics and pharmacies; local prices vary between 1 to 5 US $ per tablet. It is commonly used for respiratory tract infections, and often prescribed when all other treatments have failed (personal communication Thong Phe). No local data about macrolide use in animals are available, but a recent report from Vietnam showed that antibiotics such as macrolides, lincomycin, colistin, and aminoglycosides are actually used in livestock[41].

As the above mentioned azithromycin resistance in our study is most prevalent in *Salmonella* Choleraesuis, our findings may firstly affect empirical treatment choices for fever and presumed BSI in HIV-infected patients. Given the complex resistance patterns in *Salmonella* Choleraesuis, neither ciprofloxacin nor azithromycin appear to be safe choices; the most likely alternative in the Cambodian setting is probably a third generation cephalosporin. However, in two *Salmonella* Choleraesuis isolates the presence of ESBL was found. Extensive antibiotic resistance, including ESBL has been reported before for *Salmonella* Choleraesuis in East Asia[42, 43]. Even though ESBL prevalence in *Salmonella enterica* is still low compared to the very high rates in community-acquired *Escherichia coli* and *Klebsiella pneumoniae* isolates in the same study population[44], this is a very worrisome trend, as the potential for transmission of resistance genes is expected.

These results warrant further surveillance of resistance in invasive bacterial pathogens and *Salmonella* spp. in particular in Cambodia. More in depth research of the causes and molecular mechanisms of this in vitro measured azithromycin resistance are needed. In addition, integrated research on the human and veterinary epidemiology of *Salmonella* Choleraesuis in Cambodia is essential for better understanding of the disease dynamics and planning of public health interventions.

Conclusion

Salmonella Typhi and *Salmonella* Choleraesuis are both common *Salmonella* serovars causing BSI in Cambodian adults; *Salmonella* Choleraesuis closely associated with advanced HIV-disease. DCS and azithromycin resistance are very high in *Salmonella* Typhi and *Salmonella* Choleraesuis respectively, while presence of ESBL is emerging. Human salmonellosis has become a difficult-to-treat infection in Cambodia requiring close surveillance and public health attention.

With regards to the local treatment guidelines in SHCH, we suggest that patients with presumptive typhoid fever are empirically treated with ceftriaxone (or azithromycin in ambulatory cases), which should be subsequently adapted to ciprofloxacin or azithromycin according to the susceptibility pattern. In febrile patients with underlying HIV/AIDS however, we do not recommend the empirical use of azithromycin.

Figures and tables

Figure 1. Flow chart of *Salmonella* bloodstream infection (BSI) episodes, patients and corresponding serovars.

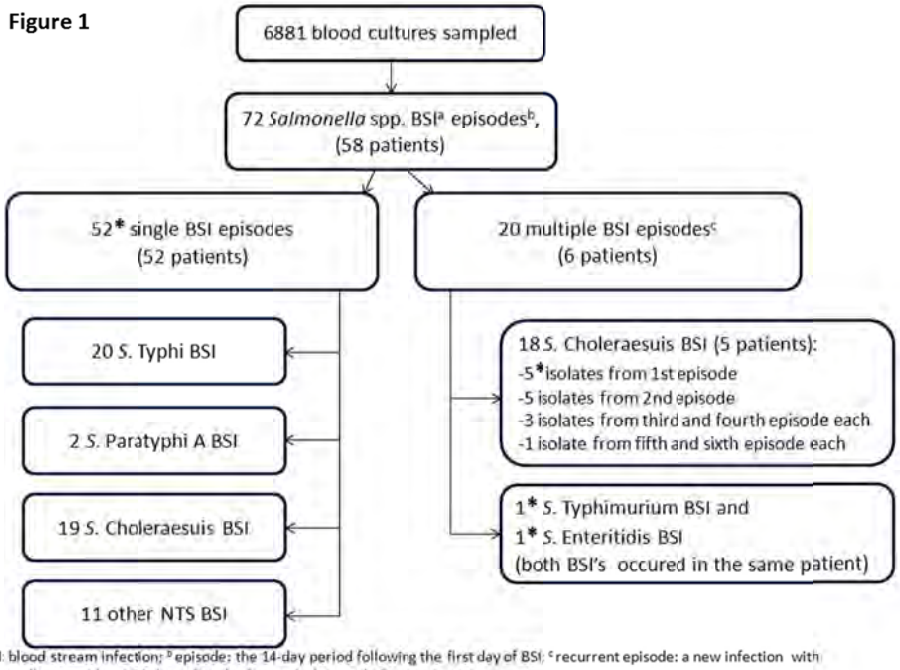
Table 1. Antibiotic resistance in 59 *Salmonella* isolates (first BSI episode only), SHCH 2007-2011.

Table 2. Distribution of minimal inhibitory concentration (MIC) for ciprofloxacin in 59 *Salmonella* isolates (first BSI episode only)

Table 3. Mutations in Gyrase and Topoisomerase and presence of qnr genes, according to serovar and resistance phenotype in 59 *Salmonella* spp.

Table 4. Distribution of minimal inhibitory concentration (MIC) for azithromycin in 59 *Salmonella* isolates.

Figure 1



[a] BSI: blood stream infection; [b] episode: the 14-day period following the first day of BSI; [c] recurrent episode: a new infection with *Salmonella* spp. at least 14 days after the former isolate and after treatment

* isolates used for resistance calculations

Table 1. Antibiotic resistance in 59 Salmonella isolates (first BSI episode only), SHCH 2007-2011.

	resistant isolates			
	(%)			(n)
Antibiotic	***S.* Typhi (n = 20)**	***S.* Choleraesuis (n = 24)**	**other NTS (n = 13)**	***S.* Paratyphi A (n = 2)**
Multi drug resistance[a]	75,0	91,7	38,5	0/2
Fluoroquinolone resistance				
Nalidixic acid	90,0	33,3	38,5	0/2
Decreased ciprofloxacin susceptibility (DCS)[b]	90,0	20,8	53,8	0/2
High level ciprofloxacin resistance[c]	0,0	0,0	7,7	0/2
Second line antibiotics				
Azithromycin[d]	5,0	70,8	15,4	0/2
Cefotaxim[e]	0,0	4,2	0,0	0/2
Combined resistance				
MDR + DCS	70,0	16,7	23,1	0/2
MDR + DCS + Azithromycin	0,0	4,2	7,7	0/2
Reserve antibiotics				
Meropenem	0,0	0,0	0,0	0/2
Tigecyclin	0,0	0,0	0,0	0/2

[a] co-resistance to ampicillin + SMX-TMP + chloramphenicol, [b] MIC ciprofloxacin > 0.064 µg/ml, see text for details, [c] MIC ciprofloxacin ≥ 4 µg/ml, [d] MIC azithromycin >16 µg/ml, [e] Not included: 1 isolate S. Choleraesuis from recurrent infection, ESBL producing

Table 2. Distribution of minimal inhibitory concentration (MIC) for ciprofloxacin in 59 *Salmonella* isolates (first BSI episode only).

	MIC ciprofloxacin (µg/ml)[a]														
Serovar (n)	0.004	0.006	0.008	0.012	0.016	0.032	0.064	0.094	0.125	0.19	0.25	0.38	6	MIC 50	MIC 90
S. Choleraesuis (24)	2	5	5	2	2	-	-	3	4	1	-	.	-	0.012	0.125
S. Paratyphi A (2)	-	-	-	1	1	-	-	-	-	-	-	-	-	NA	NA
S. Typhi (20)	-	1	-	1	-	-	-	-	1	4	10	3	-	0.25	0.38
other NTS (13)	-	-	3	-	-	-	1	1	2	2	1	2	1	0.125	0.38

NA, not applicable

[a]resistance breakpoint shown in grey

Table 5. Distribution of minimal inhibitory concentration (MIC) for azithromycin in 59 *Salmonella* isolates.

	MIC azithromycin (µg/ml)[a]																
Serovar (n)	1.5	2	3	4	6	8	12	16	24	32	48	64	96	128	>256	MIC 50	MIC 90
S. Choleraesuis (24)	1	1	3	2	-	-	-	-	-	6	1	3	2	3	2	32	128
S. Paratyphi A (2)	-	-	-	1	1	-	-	-		-	-	-	-	-	-	NA**	NA
S. Typhi (20)	-	1	6	10	2	-	-	-	-	-	-	-	1	-	-	4	6
other NTS (13)	1	2	3	4	1	-	1	-	-	-	-	1	-	-	-	4	12

NA, not applicable

[a]'epidemiological cutoff point shown in grey

Table 3. Mutations in Gyrase and Topoisomerase and presence of qnr genes, according to serovar and resistance phenotype in 59 *Salmonella*

Resistance phenotype	MIC ciprofloxacin	Serovars	n isolates	*gyr*A	*gyr*B	*par*C	*qnr*
Na^S Cip^S (n = 24)	0.004 - 0.064	S.Typhi	2	Glu133→Gly[b] (n = 2)	-	-	-
		S. Paratyphi A	2	-	-	Thr57→Ser[c] (n = 2)	-
		S. Choleraesuis	16	-	-	Thr57→Ser (n = 16)	-
		other NTS	4	Ile125→Ser[d] (n = 1)	-	-	-
Na^S DCS (n = 4)	0.125 - 0.38	other NTS	4	-	-	Thr57→Ser (n = 1)	S1 (n = 2)
Na^R DCS (n = 31)	0.094 - 0.38	S.Typhi	18	Ser83→Phe / Glu133→Gly (n = 18)	-	-	-
		S. Choleraesuis[a]	8	Ser83→Phe (n = 2)	-	Thr57→Ser (n = 7)	-
				Ser83→Tyr (n = 2)		-	-
				Asp87→Gly (n = 1)		-	-
				Asp87→Tyr (n = 1)		-	-
		other NTS	4	Ser83→Ile (n = 2)	-	-	S1 (n = 1)
				Asp87→Tyr (n = 1)		-	
Na^R Cip^R (n = 1)	6	S. Typhimurium	1	Ser83→Phe / Asp87→Asn	-	Ser80→Arg	-

Na^S, nalidixic acid susceptible; Cip^S, ciprofloxacin susceptible; Na^R, nalidixic resistant; DCS, decreased ciprofloxacin susceptibility

[a]co-presence of Ser 83(gyrA) and Thr57 (parC) mutations in 4 isolates; [b]Glu133→Gly: silent mutation; [c]Thr57→Ser: silent mutation; [d]Ile125→Ser: silent mutation

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Increase in *Salmonella enterica* serovar Paratyphi A infections in Phnom Penh, Cambodia, January 2011 to August 2013

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Abstract

We report an increased number of *Salmonella enterica* Paratyphi A infections in adults in Cambodia. Between January 2011 and August 2013, 71 Salmonella Paratyphi A isolates were recovered from blood cultures, representing a 44-fold increase compared to July 2007 to December 2010, while monthly numbers of cultures did not change. Infections with *Salmonella* Typhi increased two-fold in the same period. Most cases came from the capital Phnom Penh. These findings warrant epidemiological investigation to support public health measures.

Between 1 January 2011 and 31 August 2013, there has been a marked increase of *Salmonella enterica* serovar Paratyphi A cases diagnosed from blood cultures in Cambodian citizens, particularly in 2013, and this was reflected in an increased recovery of this pathogen from European travellers returning from Cambodia between January and August 2013 [1]. Here, we report preliminary surveillance data from Cambodia.

Background

Salmonella enterica is an important pathogen in many low and middle income countries [2]. The serovars *Salmonella* Typhi and Paratyphi cause enteric fever (*i.e.* typhoid and paratyphoid fever respectively), and are particularly prevalent in South and Southeast Asia. Treatment has become challenging because of emerging antibiotic resistance to first-line antibiotics such as chloramphenicol, ampicillin, sulphamethoxazole-trimethoprim (SMX-TMP) and more recently fluoroquinolones [3]. Over the past two decades, *Salmonella* Paratyphi A has become increasingly prevalent in Asia, causing between 15% (Pakistan, Indonesia) and 64% (Southeast China) of enteric fever cases in these countries [4]. Typhoid and paratyphoid fever are also endemic in Cambodia [5-7]. Nationwide surveillance of incidence and antibiotic resistance patterns is, however, largely lacking due to the country's very limited microbiology laboratory infrastructure [8]. Since July 2007, the Cambodian non-governmental (NGO) hospital Sihanouk Hospital Centre of HOPE (SHCH) in Phnom Penh, and the Institute of Tropical Medicine, Antwerp, Belgium co-organise surveillance of bloodstream infections in Cambodian adults attending SHCH. The 30-bed adults' hospital and its associated clinics provide over 135,000 outpatient visits and about 1,000 hospitalisations per year of patients from across Cambodia. Of 6,881 blood cultures drawn between July 2007 and December 2010, we recorded two patients infected with *Salmonella* Paratyphi A [9].

Methods

Laboratory procedures for surveillance of bloodstream infections

From all patients presenting at SHCH with signs of the Systemic Inflammatory Response Syndrome (SIRS) [10], blood cultures were taken as described in Chapter 2 and *Salmonella* identification and susceptibility testing were performed as described in the former study of this Chapter.

Case definition

In this report, 'paratyphoid fever' and 'typhoid fever' cases were defined as a patient with culture-

confirmed bloodstream infection due to *Salmonella* Paratyphi A or *Salmonella* Typhi respectively. Patients with blood cultures that did not grow after complete work-up or that revealed pathogens other than '*Salmonella* Typhi/Paratyphi A' were not considered 'cases' for the purpose of this investigation.

Ethical approval

Ethical approval was granted by the review boards at Institute of Tropical Medicine, Antwerp, the University Hospital Antwerp and the National Ethics Committee for Health Research, Phnom Penh, Cambodia respectively.

Results

Between 1 January 2011 and 31 August 2013, 102 cases of enteric fever were diagnosed in Cambodian citizens *i.e.* 71 with paratyphoid fever and 31 with typhoid fever. Seven cases were recorded in 2011, 20 in 2012 and 75 in the first eight months of 2013. As shown in Figure 1, paratyphoid fever cases were observed more frequently from April 2012 and numbers increased further from March 2013 onwards. Typhoid fever cases increased as well but to a lesser extent.

Forty-seven percent (*i.e.* 35 of 71 (49.3%) paratyphoid cases and 13/31 (41.9%) typhoid cases) were female, with a median age of 24 years (range 7–54 years); ages of paratyphoid and typhoid cases were not significantly different.

Out of 71 paratyphoid fever cases, 56 lived in the greater Phnom Penh area (Figure 2), particularly in the following districts: Russey Keo (n=10), Chamkar Mon (n=9), Tuol Kouk (n=8), Mean Chey (n=8), Dangkao (n=7), Prampir Makara (n=3), Por Senchey (n=3), Doun Penh (n=1) (Figure 3). Typhoid fever cases were more dispersed: 19 of 31 were from Phnom Penh, but distributed in small numbers among a large number of districts.

Preliminary susceptibility data based on laboratory files revealed that only 1 of 71 (1.4%) *Salmonella* Paratyphi A isolates was resistant to ampicillin, none to sulphamethoxazole-trimethoprim (SMX-TMP) and three (4.2%) displayed nalidixic acid resistance.

In contrast, 14 out of 31 *Salmonella* Typhi isolates were ampicillin resistant, 11 were SMX-TMP resistant and all but two isolates were nalidixic acid resistant. Six isolates displayed high level ciprofloxacin resistance. Results of minimal inhibitory concentrations for ciprofloxacin are pending.

Empirical treatment included most often ceftriaxone followed by oral ciprofloxacin. So far, there was neither in-hospital mortality, nor relapse recorded.

Discussion and conclusion

Between January 2011 and August 2013, and particularly in the first eight months of 2013, we noted a remarkable increase in paratyphoid fever mainly among young adults treated in our hospital and clinics in Phnom Penh. Compared to the recovery of only two *Salmonella* Paratyphi A isolates during the surveillance period 2007-2010[9], this represents a 44-fold increase, while the monthly rate of blood cultures remained constant around 150-200. In June we observed a temporary drop in cases, for which we do not have a conclusive explanation, although it may be possible that cases coincidentally visited other healthcare facilities (mostly without culture facilities) within the metropolitan area.

Although it was not possible in the present setting to calculate population-based incidence data, the clustering in time and place of the recent *Salmonella* Paratyphi A cases is of concern and suggests the implication of a common and persistent source. This could be either a continuing disseminating source (*i.e.* water) or a continuing point source such as a food vehicle. Consumption of food from street vendors has been found an independent risk factor for acquisition of paratyphoid fever in other Asian countries *e.g.* Nepal and Indonesia [13, 14] . Of note, we observed also a two-fold increase of infections due to *Salmonella* Typhi. Given the fact that predominantly adolescents and adults visit our hospital, more data on the possible spread of paratyphoid fever among Cambodian children are certainly needed.

Our findings coincide with the observation of increased numbers of S. Paratyphi A infection among European travellers from France, Germany, the Netherlands, New Zealand, Norway and the United Kingdom returning from Cambodia as communicated in a rapid risk assessment by the European Centre for Disease Prevention and Control (ECDC) on 4 September [1]. Cases from France are described in detail by Tourdjman and colleagues in this issue of Eurosurveillance [15].

Of note, most *Salmonella* Paratyphi A isolated in Cambodia between 2011 and 2013 and from travellers in 2013, displayed low resistance levels for most commonly used antibiotics in contrast with *Salmonella* Typhi and other Gram-negative pathogens in Cambodia studied between 2007 and 2010 [7]. To enable a more refined resistance description, we plan further batch-tested determination of the minimal inhibitory concentrations for ciprofloxacin, nalidixic acid and azithromycin amongst other antibiotics.

Further in-depth epidemiological research and a comparative analysis of clonal relationships between the Cambodian and European isolates are warranted to identify the source of the outbreak. Both findings, those in Cambodian citizens and European travellers, suggest that the 'hotspot' of this outbreak may be located in Phnom Penh, home to over a million inhabitants and a major gateway for visitors to the country.

Our findings were shared with the Ministry of Health of Cambodia, to allow the initiation of in-depth epidemiological investigations in order to organise the required public health measures. Cambodia, like many other low- and middle-income countries, is still building up its microbiological diagnostic capacity; across the country, less than 15 microbiology laboratories are in function [8]. In these settings, even small-scale clinical laboratories, such as the one in our hospital, may play an important role as 'sentinel' for emerging pathogens and resistance patterns.

Figures

Figure 1. *Salmonella* Paratyphi A and *Salmonella* Typhi infections diagnosed at Sihanouk Hospital Centre of HOPE, Phnom Penh, Cambodia, January 2011–August 2013 (n=102)

Figure 2. Geographical origin of *Salmonella* Paratyphi A cases diagnosed at Sihanouk Hospital Centre of HOPE, Phnom Penh, January 2011-Aiugust 2013 (n = 71)

Figure 3. Distribution of *Salmonella* Paratyphi A cases living in Phnom Penh, diagnosed at Sihanouk Hospital Centre of HOPE Phnom Penh, Cambodia, January 2011–August 2013 (n=56a)

FIGURE 1

Salmonella Paratyphi A and *Salmonella* Typhi infections diagnosed at Sihanouk Hospital Centre of HOPE, Phnom Penh, Cambodia, January 2011–August 2013 (n=102)

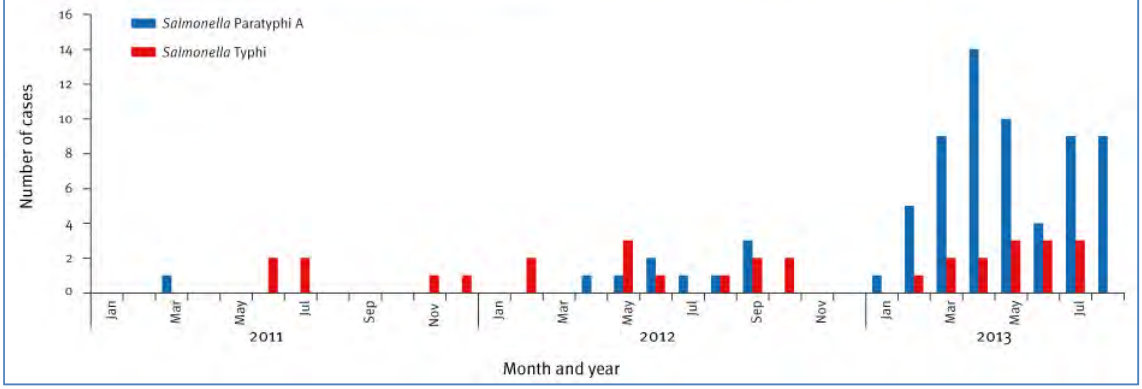

FIGURE 2

Geographical origin of *Salmonella* Paratyphi A cases diagnosed at Sihanouk Hospital Centre of HOPE, Phnom Penh, Cambodia, January 2011–August 2013 (n=71)

FIGURE 3

Distribution of *Salmonella* Paratyphi A cases living in Phnom Penh, diagnosed at Sihanouk Hospital Centre of HOPE, Phnom Penh, Cambodia, January 2011–August 2013 (n=56[a])

[a] District unknown for seven cases.

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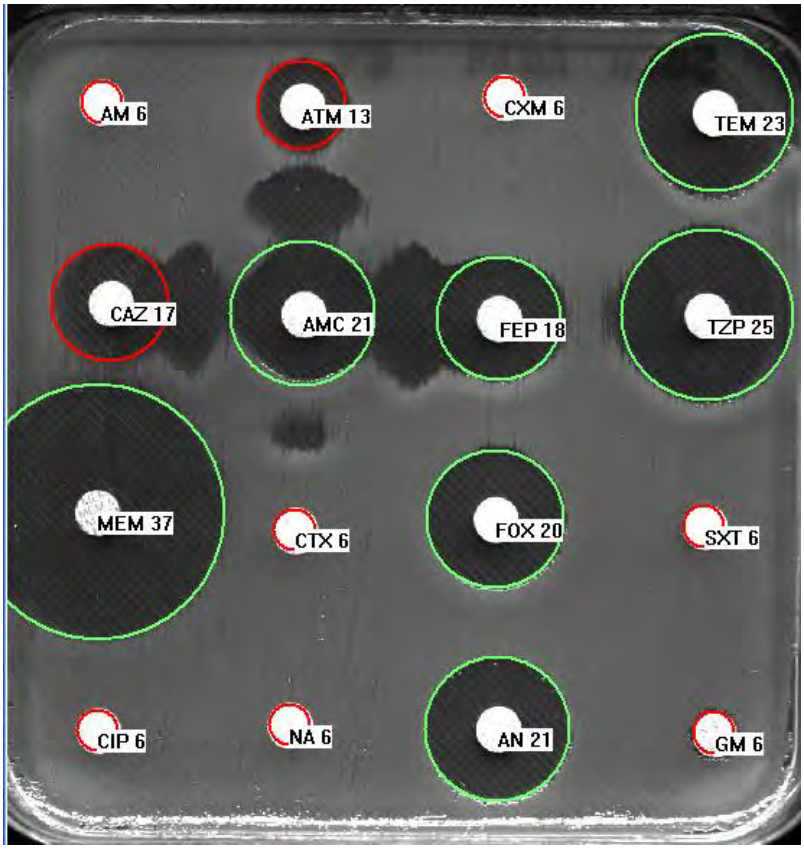
Chapter 5
Bloodstream infections due to *Enterobacteriaceae*: Pandora's box of resistance.



On June 11th 2010, a 68-year old women with known liver cirrhosis was hospitalised with cholangitis due to an obstructing stone in the common bile duct. Blood cultures revealed she had also *Escherichia coli* BSI. The susceptibility pattern (picture) revealed resistance to amoxicillin (AM), aztreonam (ATM), cefuroxim (CXM), ceftazidime (CAZ, cefotaxime (CTX), sulphamethoxazole-trimethoprim (SXT), ciprofloxacin (CIP), nalidixic acid (NA) and gentamicin (GM). The pathogen was found to carry the ESBL's CTX-M-15 and CTX-M-27 and the beta-lactamases TEM and oxa 1/30. After initial inappropriate empirical treatment, the patient survived this episode thanks to surgery and a 11-day treatment course with meropenem.

These invasive infections with *Enterobacteriaceae* containing numerous resistance genes -like 'Pandora's boxes'- turned out to be very common in patients attending SHCH.

Prevalence and distribution of beta-lactamase coding genes in third generation cephalosporin-resistant *Enterobacteriaceae* from bloodstream infections in Cambodia.

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Abstract

Background

Resistance to third generation cephalosporins in Gram-negative bacteria is spreading rapidly in Asia. We describe the prevalence and distribution of extended spectrum beta-lactamase (ESBL), AmpC beta-lactamase and carbapenemase coding genes in cefotaxime-resistant *Enterobacteriaceae* isolates from bloodstream infections (BSI) in Cambodia.

Methods

All *Enterobacteriaceae* isolated from BSI in adult patients at Sihanouk Hospital Centre of HOPE, Phnom Penh, Cambodia (2007-2010) were evaluated. Antimicrobial susceptibility testing by disk diffusion and MicroScan (Siemens, Germany) according to CLSI guidelines. Screening for ESBL, plasmidic AmpC and carbapenemase coding genes was performed by multiplex PCR sequencing assays. Identification of the ST 131 clone was performed in all CTX-M-positive *Escherichia coli*, using PCR targeting the papB gene.

Results

Out of 183 *Enterobacteriaceae* from BSI, 91 (49.7%) isolates (84 episodes, 83 patients) were cefotaxime-resistant (68 *Escherichia coli*, 17 *Klebsiella pneumoniae*, 6 *Enterobacter* spp.). Most episodes were community-acquired (66/84; 78.3%).

ESBLs were present in 89/91 cefotaxime-resistant isolates (97.8%) of which 86 (96.6%) were CTX-M, mostly CTX-M-15 and CTX-M-14. CTX-M-15 was frequently associated with TEM and/or OXA-1/30 coding genes and with phenotypic co-resistance to ciprofloxacin, sulphamethoxazole-trimethoprim and gentamicin (39/50, 78.0%). Plasmidic AmpC (CMY-2 and DHA-1 types) were found alone (n = 2) or in combination with ESBL (n = 4). Eighteen *E. coli* were identified as B2-ST 131-O25B of which 11 (61.1%) carried CTX-M-14. No carbapenemase resistance was detected.

Conclusion

Third generation cephalosporin resistance among *Enterobacteriaceae* from BSI in Cambodia is common, and is predominantly associated with CTX-M-15 and CTX-M-14. These findings warrant urgent action on containment of antibiotic resistance in Cambodia.

Introduction

Resistance to third generation cephalosporins in Gram-negative bacteria has been associated with increased healthcare costs and higher rates of inappropriate therapy and mortality [1, 2]. The predominant underlying mechanism is the presence of extended spectrum beta-lactamase (ESBL), mainly of the CTX-M, SHV or TEM-type. Over the past decade, CTX-M has become the most prevalent ESBL-type worldwide [3].

High rates of ESBL-positivity among *Escherichia coli*, *Klebsiella* species and other *Enterobacteriaceae* have been described across Asia, both in hospital and in community settings [4]. CTX-M, particularly CTX-M-15 and CTX-M-14 have been reported as the most common ESBL types in the Indian subcontinent, Southeast Asia and East Asia, although local patterns may differ and minor ESBL-types (*e.g.* VEB) are also circulating in certain areas [5]. Common risk factors for the acquisition of ESBL-producing strains in Southeast Asia include previous exposure to antibiotics (particularly cephalosporins and fluoroquinolones) and recent hospitalization or nosocomial setting [6, 7].

For Cambodia, data on prevalence, type and mechanisms of ESBL are scarce. In a prospective cohort study of patients with urinary tract infection, Ruppé and coworkers reported the presence of ESBL -all of the CTX-M type- in 37.7% of *E. coli* from 93 urinary samples [8].

In a recent study in Cambodian adults, we described the predominance of Gram-negative bacteria (mostly *E. coli*) in bloodstream infections (BSI), with about 50% resistance rate to third generation cephalosporins in *Enterobacteriaceae*, associated with high rates of co-resistance to fluoroquinolones, sulphamethoxazole-trimethoprim (SMX-TMP) and gentamicin [9]. Here we report the mechanisms underlying these complex resistance patterns in *Enterobacteriaceae* isolated from Cambodian adult patients with BSI.

Material and methods

Microbiological data

Between 2007-2010, blood cultures were taken as described in Chapter 2. Isolates were stored at -70°C on porous beads in cryopreservative (Microbank, Pro-Lab Diagnostics, Richmond Hill, Canada), subsequently retrieved and shipped to Institute of Tropical Medicine (ITM), Antwerp (September 2010 and January 2011), where identification and susceptibility testing were repeated with MicroScan (Siemens, Germany) according to CLSI guidelines (M100-S22) [11]. All cefotaxime-resistant isolates from this collection were then referred to the National Reference Laboratory (Centre

Hospitalier Universitaire Dinant-Godinne UCL) for confirmation of ESBL expression and genotypic characterization of the isolates.

In the Reference laboratory, bacterial identification was confirmed using MALDI-TOF mass spectrometry (Bruker, Leipzig, Germany), and antimicrobial susceptibility testing was carried out by disk diffusion according to CLSI guidelines (M100-S22)[11]. The presence of ESBL and/or plasmidic AmpC (pAmpC) was suspected on the basis of the phenotypic resistance patterns and was confirmed phenotypically by combination disc test between different cephalosporins indicator substrates (cefotaxime, ceftazidime) and different inhibitors (clavulanate, phenylboronic acid).

Screening for genes encoding conventional ESBL (CTX-M of group 1, 2 and 9, SHV, TEM), minor ESBL (VEB, PER, BEL, GES), pAmpC and carbapenemase (VIM, IMP, NDM, OXA-48, KPC) was done by an end-point multiplex polymerase chain reaction (PCR) using a set of four validated (ISO15189 standard) assays[12 13] followed by sequencing of all CTX-M genes detected. Sequence homology was determined using the BLASTX search tool using a non-redundant protein sequences' database and comparison with the Lahey database (http://www.lahey.org/studies/).

Identification of the ST 131-clone was performed in the CTX-M-positive *E. coli*, using PCR targeting the papB gene [14].

Clinical data and definitions

Basic clinical and epidemiological data were collected in all patients with presumed BSI during the prospective surveillance study. Infections were considered 'nosocomial' if they occurred more than two days after hospitalization and 'community-acquired' if starting before or during the two first days of hospitalization. Outcome was assessed at discharge from the hospital.

Statistical analysis

Associations were assessed using Fisher's exact test and considered statistically significant at p-values < 0.05. Data were analyzed using Stata version 10.2 (Stata Corp, College Station, Texas, USA) and Excel 2003 (Microsoft Corporation, Redmond, Washington, USA).

Ethical considerations

Ethical approval was granted from the review boards at the Institute of Tropical Medicine, Antwerp, the University Hospital Antwerp and the National Ethics Committee for Health Research, Phnom Penh, Cambodia. In SHCH, blood cultures are part of the standard diagnostic work-up of patients with a suspicion of bacteremia. For clinical and demographic data the standard information present on the laboratory request form was used. Patients were identified with a unique hospital number while the anonymity status of the patients to any third party was preserved and guaranteed during and after the study.

Results

Clinical and demographic data

Out of 183 non-duplicate *Enterobacteriaceae* isolated from blood cultures, 91 isolates (from 84 BSI episodes in 83 patients) were cefotaxime-resistant (Figure 1), and will be further discussed in this chapter. These isolates included *Escherichia coli* (n = 68), *Klebsiella pneumoniae* (n = 17), *Enterobacter cloacae* (n = 5) and *Enterobacter kobei* (n = 1). Seven patients had a polymicrobial BSI with two different *Enterobacteriaceae* isolated.

Of all 91 patients infected with cefotaxime-resistant isolates, 59.0% were women, with a median age of 47 years (17-78 y); they came from 14 different provinces of Cambodia, predominantly the capital region and adjacent southeastern provinces. Main co-morbidities were infection with the human immune deficiency (HIV)-virus (n=20; 24.1%), chronic liver disease (n=18; 21.7%), chronic renal disease (n=12; 14.5%) and diabetes mellitus (n=13; 15.7%).

The primary sources at the origin of the BSI could be clearly identified in 56 out of 84 episodes (66.7%), and were mainly urogenital (n=29; 51.8%) and intra-abdominal infections (n=18; 32.1%) besides respiratory tract infections (n=5; 8.9%) and skin and soft tissue infections (n=4; 7.1%). Sixty-six of the 84 (78.6%) BSI episodes were community-acquired. Exposure to antimicrobial therapy in three months prior to blood culture sampling was found in 42.9% (n=36) of the cases.

Patient outcome could be assessed in 63 (75.0%) episodes: 20 patients (31.7%) died, three (4.8%) were discharged for palliative care at home and four (6.3%) were referred to another hospital. The remaining 36 patients made a full recovery.

Phenotypic resistance patterns

As shown in Table 1, co-resistance to non-beta lactam antibiotics was frequently found, in particular for ciprofloxacin, SMX-TMP and gentamicin. Combined resistance to these three antibiotics in addition to cefotaxime resistance occurred in up to 61.5% (56/91) of the isolates, particularly in *E.coli*. In contrast, we did not observe resistance to meropenem and amikacin, and found low levels of resistance to colistin (4/91, 4.4%).

Resistance mechanisms

As shown in Figure 1, ESBL as unique resistance mechanism to beta-lactams was found in 85/91 (93.4%) isolates, plasmidic AmpC (pAmpC) in 2 of 91 (2.2%) and a combination of ESBL and pAmpC in

4 out of 91 (4.4%). In line with the phenotypic findings, no carbapenemase-coding genes were present in any of the isolates.

The frequency of the different ESBLs, CTX-M groups and gene variants is shown in Table 2. Bla_{CTX-M}, present in 86 of 89 ESBL-positive isolates (96.6%), was by far the most common resistance encoding gene. Most CTX-M types belonged to CTX-M-group 1 (n = 50) or to CTX-M-group 9 (n = 30); six isolates carried a combination of genes from CTX-M- group 1 and group 9. CTX-M-15 (n = 41) and CTX-M-14 (n = 21) were the most common types. Plasmidic Amp C included CMY-2-like in four *E. coli* and DHA-1 in two *K. pneumoniae* isolates respectively. Figure 2 displays the prevalence evolution of the different CTX-M groups over the study period.

The association of several beta-lactamase encoding genes (*i.e.* bla_{TEM}, bla_{SHV} or $bla_{oxa\ 1/30}$) was frequent. The most common resistance pattern observed was the combinations of CTX-M-15 and OXA-1/30 (18/91; 19.8%); in 15 of those isolates combined resistance to ciprofloxacin, SMX-TMP and gentamicin was also present. As shown in Table 3, co-resistance to non-betalactam antibiotics was found more frequently in isolates with CTX-M of group 1 (39/50; 78.0%) as compared to those positive for CTX-M of group 9 (14/30; 46.7%, p = 0.007).

No significant differences in antibiotic exposure, nosocomial infection rates or mortality was observed between patients with isolates carrying CTX-M of group 1 versus CTX-M of group 9 (data not shown).

Presence of E. coli ST 131

Out of 67 CTX-M-positive *E. coli* tested, 18 (26.9%) were found to be of the B2-ST 131-O25B type. Figure 3 displays the evolution of its proportion over time, with a gradual absolute and proportional increase of *E. coli* ST 131 over time. Of note, isolates of the ST 131 type were more frequently carrying CTX-M of group 9 (15/18, 83.3%) as compared to 10 out of 49 (20.4%) non-ST 131 *E. coli*, p < 0.001); this was particularly the case for CTX-M-14 (11 out of 18 *E. coli* ST 131; 61.1%). In contrast, group 1 CTX-M genes were significantly more frequent in non-ST 131 isolates (36/49 (73.5%) versus 3 in 18 (16.7%) ST 131 *E. coli* isolates, p < 0.001).

Besides these associations, infections caused by *E. coli* of the B2-ST 131-O25B type were not significantly different from those caused by non-ST 131 *E. coli* in terms of patients' age, gender, co-morbidity, infection focus, co-resistance to non-beta-lactam antibiotics or outcome.

Discussion

In this study we found that resistance to third-generation cephalosporins among *Enterobacteriaceae* was widespread in Cambodia and was mostly associated with a CTX-M type ESBLs, either alone or in combination with other beta-lactamases and occasionally including pAmpC (*i.e.* CMY-2-like type in *E. coli* and DHA type in *K. pneumoniae*). We observed a clear predominance of CTX-M-15 and of CTX-M-14, but also a wide variety of genetic patterns, including combinations of two different CTX-M-types. The presence of an ESBL was very often associated with co-resistance to ciprofloxacin, SMX-TMP and gentamicin, especially in isolates of CTX-M-15 type. This is consistent with previous studies which showed the co-localization of these different resistance genes including the one coding for CTX-M-15 on the same plasmids [3].

The overall findings are in line with data from other Asian countries [4]. A recent surveillance study of 699 invasive *Enterobacteriaceae* from 11 countries in the Asian-Pacific region [15] and other studies confirmed high prevalence rates of CTX-M in the region, with predominance of CTX-M-15 on the Indian subcontinent [16], Singapore, Malaysia and the Philippines [17] whereas CTX-M-14 was most frequently found ESBL in China [18], South Korea [19] and Taiwan [20]. Besides CTX-M, SHV is also a common ESBL in Asia, and presence of non-ESBL mechanisms *e.g.* pAmpC (CMY, DHA) does also frequently occur in India, Taiwan, South Korea and Vietnam [15].

In Thailand, SHV was the dominant ESBL until the late 1990's, when CTX-M14, CTX-M-15 and CTX-M-55 became the most common ESBL[21, 22]. CTX-M-14 is particularly prevalent in East Asia, although CTX-M-15 has also in this area spread recently. For instance, a 7-year surveillance study in Taiwan (2001-2007) observed, besides predominance of CTX-M-14, a ten-fold increase in the prevalence of CTX-M-15 over the study period [20]. While the presence of two or more ESBL-coding genes is widespread, the co-presence of genes encoding two different CTX-M's is less common, and predominantly reported in Korea, often from a nosocomial context [19, 23, 24]; this was not the case in all our double-CTX-M-isolates.

In our study population, pAmpC occurred sporadically without a clear evolution in time. In other Asian settings the co-presence of CTX-M and pAmpC has been found more commonly *e.g.* in 50-80% of ESBL-positive isolates in India [25], in 33% of the Singapori CTX-M positive *Enterobacteriaceae* [26], while in Thailand the combination of CTX-M and pAmpC occurs in nearly 70% of less common *Enterobacteriaceae*, which was found to jeopardize frequently the diagnosis of ESBL [27] .

Taken together, the observed abundance of genes and their combinations on the Asian continent reflects not only the existing biodiversity but also high rates of antibiotic pressure inducing further

resistance genes and multiple occasions for their transfer between bacterial species, human and animal hosts and within the larger environment.

In the present study, we noted the emergence of the ST 131 *E. coli* clone, which was closely associated with CTX-M-14. This is in contrast with a large number of reports on the globally emerging, multi drug resistant CTX-M-15-positive ST 131 *E. coli* [3, 28, 29]. However, recent surveillance data from Japan describe the association of the ST 131-clone with CTX-M-14 [30], and more recently with CTX-M-27 and to a lesser extent with CTX-M-15 [31, 32]. In China, *E. coli* ST 131 was found in association with either CTX-M-14 and CTX-M-15 [18] whereas in *E. coli* from neighboring Laos, ST 131 associated with CTX-M-14 is quickly emerging, next to other sequence types such as ST 648 and ST 405[33]. Together with these recent publications, our findings may help to refine the picture of *E. coli* ST 131 in Asia, of which the knowledge is still limited [34]; its recent epidemiology being probably the resultant of the vertical (clonal) spread of successful clones and horizontal (between pathogens) spread of resistance genes through plasmids transfer within and across different species [35]. Subsequent shuffling and recombination of genes in the same or in different plasmids could explain diversification over time. Also, these findings from invasive isolates are but the tip of the iceberg and a better understanding of the entire *E. coli* epidemiology will require more data on presence of ST 131 in other patient groups, healthy carriers, pet and food animals and environment at large.

Finally, in spite of the earlier described association of the ST 131-clone with increased clinical severity [3], we found no evidence for this among our patients. The absence of a different outcome in patients infected by a ST 131-*E. coli* as compared to infections due to non-ST 131 isolates has also recently been described in a patient series from South-Korea [36]. However, measuring and interpreting virulence in *E. coli* infections is very complex [37] and the assessment of its clinical impact requires probably large numbers of more refined patient data with sufficiently long follow-up.

The overall impression remains that with regards to the epidemiology of third-generation cephalosporin resistance in Asia, the globally observed trends do apply, but particular trends and a wide genetic heterogeneity can be found in each country and region.

Our study had several limitations. The number of isolates was limited and collected at a single center over a relatively short period. Long-term surveillance of isolates from multiple sites in Cambodia (including rural and first line health care settings) is needed to confirm the trends we observed in our study. In addition, information on presence of other (non-ST 131) sequence types in *E. coli* and other *Enterobacteriaceae* [38] would be welcome for in-depth understanding of the local epidemiology and the detection of novel trends. Next, given the high rates of co-morbidity and prior antibiotic use in

our study population, our results may have overestimated resistance patterns present in the community. However, as was shown in a Thai study on fecal flora of healthy volunteers, ESBL carriages rates in the community may be as high as 60%[39]. Finally, not for all patients full clinical information was available; prospective clinical studies in parallel with surveillance studies are needed to assess the clinical context and impact of the newly observed resistance patterns.

Nevertheless, the present data represent -at the best of our knowledge- the first detailed description in Cambodia of resistance mechanisms in systematically collected *Enterobacteriaceae* from bloodstream infections.

Our data were collected within the framework of a capacity building program focusing on bacterial resistance surveillance and antibiotic stewardship. The findings have generated awareness on the presence and extent of Gram-negative resistance at SHCH and in Cambodia and they have been integrated in training modules for other hospital teams in the country. In addition, the combined collection of microbiological and clinical data was felt particularly useful in understanding the epidemiological and clinical value of the findings.

At a larger scale, our findings highlight the urgent need for a nationwide surveillance in Cambodia. This requires a network of well-functioning laboratories and diagnostic cultures affordable for patients and health systems, which have been largely lacking for decades in Cambodia (and other low-resources settings)[40]. The recent introduction of blood culture facilities in several provincial and public hospitals by a consortium of international and national partners is hopefully a first step towards the creation of this much needed bacterial surveillance system [40].

From a clinical point of view, high rates of complex resistance imply access to good quality and affordable broad spectrum antibiotics (*e.g.* carbapenems and amikacin) for effective treatment of the severely ill, as these drugs are largely lacking or unaffordable in Cambodian hospitals. However, wise positioning of these broad spectrum antibiotics into standard treatment guidelines is a difficult exercise; more information from clinical studies is also needed to identify those most in need of broad spectrum antibiotics.

The observed complex combinations of resistance genes suggest intense antibiotic pressure, as was confirmed by high rates of reported prior antibiotic use. This highlights an urgent need for surveillance of antibiotic use by Cambodian people and in their livestock, in particular for antibiotic with high risk on 'collateral damage' such as cephalosporins, fluoroquinolones and carbapenems [41] in order to halt the further increase of resistance.

Conclusion

We found extensive and complex resistance patterns in *Enterobacteriaceae* with cefotaxime resistance, due to high prevalence of CTX-M-14, CTX-M-15 type ESBL's and other beta-lactamases. *Enterobacteriaceae* seem to have become genuine 'Pandora's boxes': filled with resistance genes and spreading quickly around the world. These findings warrant urgent action on containment of antibiotic resistance in Cambodia.

Figures and Tables

Figure 1. Study flowchart

Figure 2. Evolution of CTX-M types over time

Figure 3. Prevalence of *E. coli* ST 131 over time

Table 1. Phenotypic resistance in 91 cefotaxime-resistant *Enterobacteriaceae*, Cambodia (2007-2010).

Table 2. Resistance mechanisms underlying cefotaxime resistance in 91 *Enterobacteriaceae* isolates from BSI, Cambodia.

Table 3. Patterns of combined resistance in 89 ESBL-positive *Enterobacteriaceae* (per ESBL genotype)

Figure 1. Study flowchart

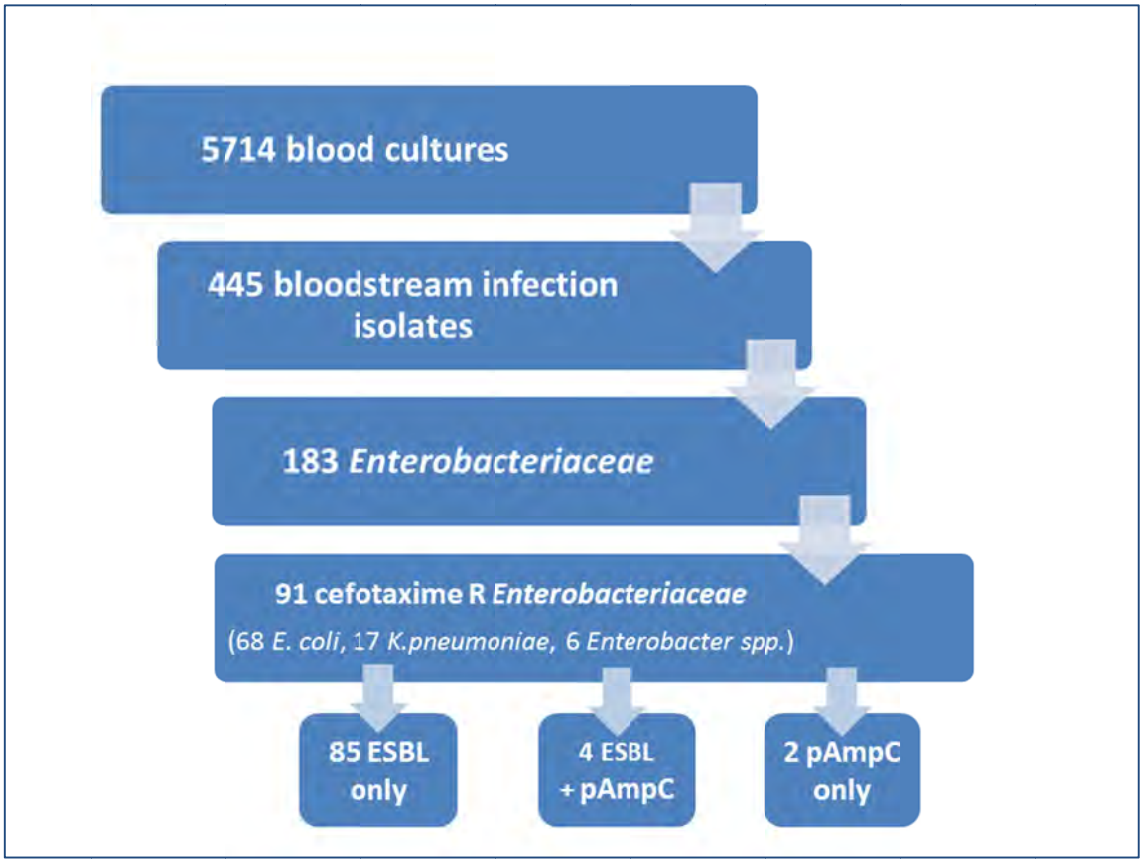

Figure 2a. Evolution of CTX-M type extended spectrum beta-lactamases (ESBL) over time. (ceph3: third generation cephalosporin; X-axis displays calendar years and Y-axis absolute n of *Enterobacteriaceae* isolates)

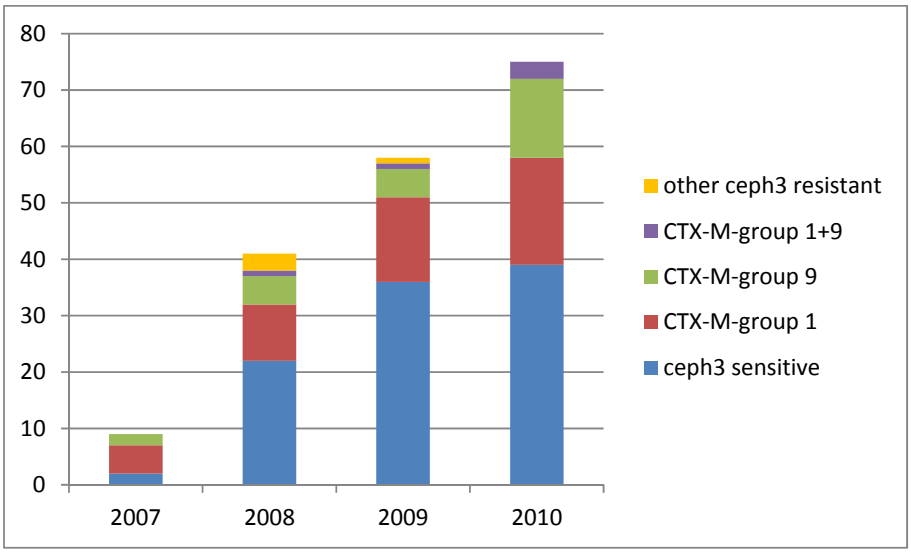

Figure 3. Prevalence of ESBL *E. coli* ST 131 over time (X-axis displays calendar years, Y-axis absolute n of different groups of *E. coli* isolates)

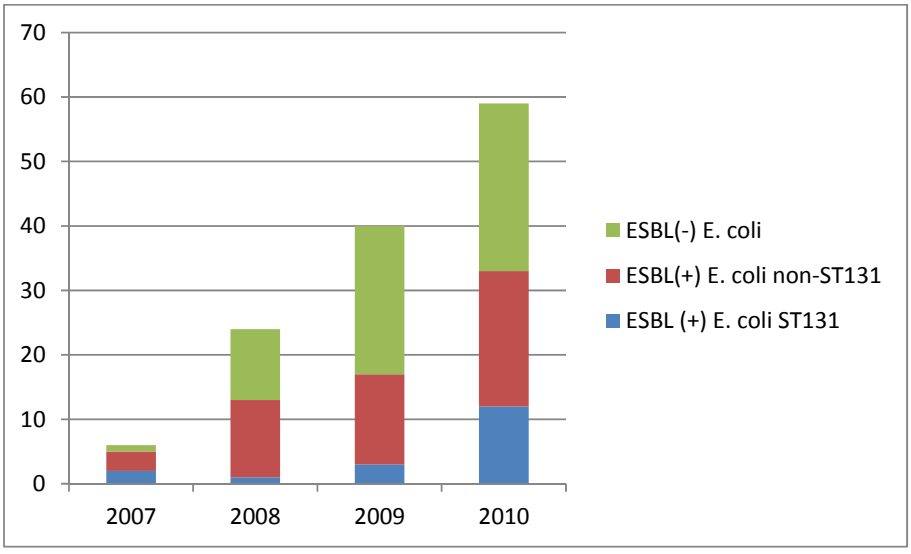

Table 1. Phenotypic resistance[1] in 91 cefotaxime-resistant *Enterobacteriaceae*, Cambodia (2007-2010).

	E. coli (n = 68)	%	*K. pneumoniae* (n = 17)	%	*Enterobacter* spp. (n = 6)	%
ceftazidime	39	57,4	6	35,3	5	83,3
cefepime	23	33,8	2	11,8	1	16,7
amoxicillin-clavulanic acid	14	20,6	2	11,8	5	83,3
piperacillin-tazobactam	6	8,8	3	17,6	1	16,7
ciprofloxacin	63	92,6	8	47,1	6	100,0
SMX-TMP[2]	65	95,6	15	88,2	5	83,3
gentamicin	52	76,5	10	58,8	5	83,3
amikacin	0	0,0	0	0,0	0	0,0
aztreonam	39	57,4	5	29,4	2	33,3
meropenem	0	0,0	0	0,0	0	0,0
colistin	1	1,5	0	0,0	3	50,0
ciprofloxacin + SMX-TMP	60	88,2	7	41,2	5	83,3
ciprofloxacin + SMX-TMP + gentamicin	47	69,1	5	29,4	4	66,7

[1]as assessed by disk diffusion except for colistin which was assessed by Microscan; [2]SMX-TMP: sulphamethoxazole-trimethoprim

Table 3. Patterns of co-resistance to non-beta lactam antibiotics in 89 ESBL-positive *Enterobacteriaceae* (per ESBL genotype)

Antibiotics	Total (n = 89)	CTX-M-group 1 (n = 50)	CTX-M-group 9 (n = 30)	CTX-M-group 1 + 9 (n = 6)	non-CTX-M ESBL (n = 3)
ciprofloxacine	76	48 (96.0%)	22 (73.3%)	4	1
ciprofloxacin + SMX-TMP	72	47 (94.0%)	21 (70.0%)	3	1
ciprofloxacin + SMX-TMP + gentamicin	56	39 (78.0%)	14 (46.7%)	3	0
ciprofloxacin + SMX-TMP + gentamicin + amikacin	0	0 (0.0%)	0 (0.0%)	0	0
ciprofloxacin + SMX-TMP + gentamicin + colistin	1	1 (2.0%)	0 (0.0%)	0	0

Table 2. Resistance mechanisms underlying cefotaxime resistance in 91 *Enterobacteriaceae* isolates from BSI, Cambodia.

ESBL (n = 89)		TEM	SHV	OXA-1/30	n isolates	+ pAmpC	+CipCotGe[6]	*E. coli* ST 131
CTX-M-group 1 (n = 50)[1]								
	CTX-M-15 (n = 41)	-	-	+	18	1 (CMY-2-like)	15	2
		+	-	-	6	-	3	-
		+	-	+	9	-	8	1
		+	+	+	5	-	4	-
		-	-	-	2	1 (CMY-2-like)	0	-
		+	+	-	1	-	1	-
	CTX-M-55 (n = 7)	+	-	-	6	1 (CMY-2-like)	5	-
		-	-	-	1	-	1	-
	CTX-M-3 (n = 2)	+	-	-	1	-	1	-
		+	+	-	1	-	0	-
CTX-M-group 9 (n = 30)[2]								
	CTX-M-14 (n = 21)	+	-	-	8	-	6	4
		-	-	-	8	-	5	7
		+	+	-	3	-	0	-
		-	+	-	1	-	0	-
		-	-	+	1	-	0	-
	CTX-M-27 (n = 9)	+	-	-	4	-	2	1
		-	-	-	4	-	1	3
		+	+	-	1	-	0	-
CTX-M-group 1 + group 9 (n = 6)[3]								
	CTX-M-15 + CTX-M-14 (n = 2)	+	+	-	1	1 (DHA)	1	-
		-	-	+	1	-	0	-
	CTX-M-15 + CTX-M-27 (n = 3)	-	-	+	1	-	1	-
		-	-	-	1	-	0	-
		+	-	+	1	-	1	-
	CTX-M-3 + CTX-M-27 (n = 1)	+	+	-	1	-	0	-
non-CTX-M ESBL (n = 3)[4]		+	+	-	2	-	0	-
		-	+	-	1	-	0	-
non-ESBL (n = 2)[5]		+	-	-	1	1 (CMY-2-like)	0	-
		+	+	+	1	1 (DHA)	0	-

[1]includes *E. coli* (n = 39), *K. pneumoniae* (n = 6) and *Enterobacter* spp. (n = 5); [2]includes *E. coli* (n = 25) and *K. pneumoniae* (n = 5); [3]includes *E. coli* (n = 3) and *K. pneumoniae* (n = 2) and *Enterobacter*sp. (n = 1); [4]includes *K. pneumoniae* (n = 3); [5]includes *E. coli* (n = 1) and *K. pneumoniae* (n = 1); [6]CipCotrGe: combined resistance to cefotaxime, ciprofloxacin, SMX-TMP and gentamicin

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Escherichia coli bloodstream infections in Cambodian adults: risk factors for acquisition of extended spectrum beta-lactamases and mortality.

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Abstract

Background

The emergence of extended spectrum beta-lactamases (ESBL) limits treatment choices for *Escherichia coli* bloodstream infections (BSI), particularly in resource limited settings. We retrospectively studied a cohort of adults with *E. coli* BSI in Phnom Penh, Cambodia (July 2007 to December 2010) for risk factors associated with ESBL-producing *E. coli* (ESBL-EC) and mortality.

Methods

Blood was cultured from patients suspected of BSI. Testing for ESBL was performed by multiplex PCR followed by direct sequencing. Clinical data were retrieved from patients' charts. Relative risks were assessed in univariate and multivariate analysis.

Results

A total of 126 patients were identified with *E. coli* BSI (62.7% women, median 51 years (23-81 y). Common foci were urosepsis (n = 48; 38.1%), spontaneous bacterial peritonitis (n = 16; 12.7%) and biliary tract infection (n = 14; 11.1%). ESBL-EC accounted for 65 (51.6%) BSI . Prior antibiotic use (RR 1.46 (1.03-2.09), p =0.035), particularly cephalosporin use (RR 1.59 (1.23-2.05), p < 0.001) were independent risk factors for ESBL-positive infection. Thirty-two (29.1%) patients died. Main independent risk factor for mortality was a Charlson's comorbidity index of ≥ 2 (RR 2.75 (1.11 – 6.81), p=0.028) besides trends to higher mortality in presence of shock (RR 1.58 (0.91-2.77), p = 0.105) and ESBL (RR 1.93 (0.95-3.92), p = 0.071) but not for inappropriate empirical treatment (RR 1.16 (0.66-2.06), p = 0.669).

Conclusions

E. coli BSI in Cambodian adults has a high mortality which is strongly associated with comorbidity, and to a lesser extent with severity of illness and presence of ESBL

Introduction

E. coli bloodstream infections (BSI) occur frequently worldwide, both as community- and health care-associated infections, and they may cause considerable mortality and morbidity [1, 2]. Traditional antibiotic treatment of these infections includes -depending on the underlying infection focus- beta-lactam drugs (*i.e.* penicillins or cephalosporins) or fluoroquinolones. The global emergence of extended spectrum beta-lactamases (ESBL) and other mechanisms causing complex resistance patterns in Gram-negative pathogens has led to drastic changes in the medical management of these infections, with increased use of carbapenems, aminoglycosides and other broad spectrum antibiotics. The occurrence of infections caused by ESBL-producing organisms has also been associated with increased healthcare costs, inappropriate therapy and higher morbidity and mortality rates [3, 4].

Risk factors associated with the acquisition of ESBL *E. coli* bloodstream infection include older age, prior exposure to antibiotics (particularly cephalosporins and fluoroquinolones), health-care associated or nosocomial infections, presence of a urine catheter or other genitourinary tract procedures [5, 6]. However, due to the rapidly evolving epidemiology and the high prevalence of ESBL-asymptomatic carriage in the community, risk factors may often be lacking [7]. For several industrialized countries, recent travel to a country with high ESBL-prevalence (*e.g.* India, Egypt) was found an independent risk factor for colonization and infection with an ESBL-positive pathogen [8, 9].

In the past two decades, Asia has become a particular hotspot for complex Gram-negative resistance [10]; this has been described increasingly in the Indian subcontinent, Southeast Asia and the Asia-Pacific area [11, 12]. Of particular concern is the fast emergence of ESBL of the CTX-M-15 genotype, which has been associated with co-resistance to non-beta lactam antibiotics and clonal pandemic spread through association with the emerging and virulent clone *E. coli* B2-ST 131-025b:H4[13].

In a recent blood culture-based prospective survey of adults admitted to the Sihanouk Hospital Centre of HOPE (SHCH), Phnom Penh, Cambodia, *E. coli* represented the single most prevalent pathogen, causing about 30% of all BSIs [14]. Nearly 50% of the *E. coli* isolates displayed resistance to third-generation cephalosporins. The underlying mechanism was ESBL in >90% of isolates *i.e.* predominantly CTX-M of group 1 and group 9, often combined with various other beta-lactamases also occasionally including plasmidic AmpC [15]. Given the treatment implications, and in order to better understand the underlying risk for developing ESBL-positive *E. coli* BSI and/or having an adverse (clinical) outcome, we undertook a retrospective cohort analysis of all consecutive patients with *E. coli* BSI attending SHCH between July 1st 2007 and December 1st 2010.

Material and methods

Between 2007 and 2010, blood cultures were taken from patients with SIRS[16] as described in Chapter 2. *E. coli* isolates were stored locally at -70°C and were subsequently sent to the Institute of Tropical Medicine (ITM) for confirmation of identification and susceptibility patterns using MicroScan (Siemens Healthcare Diagnostics, Deerfield, USA). Interpretative breakpoints were those defined by the 2012 Clinical and Laboratory Standards Institute (CLSI)[17]. Double combination disk test with ceftazidime, cefepime, ceftazidime-clavulanic acid and cefepime-clavulanic acid as described elsewhere [18] was used for phenotypic confirmation of ESBL on strains with intermediate susceptibility or resistance to cefotaxime.

All isolates displaying resistance to any third generation cephalosporin agent were shipped to the National Reference Laboratory for monitoring of Antimicrobial Resistance (Centre Hospitalier Universitaire Dinant-Godinne, UCL ,Belgium) for further analysis as described earlier in this Chapter.

Clinical data and definitions

Clinical and epidemiological data were retrieved from the laboratory request form and through retrospective review of the patients' charts.

Infections were considered 'nosocomial' (hospital onset) if they occurred more than two days after hospitalization and 'community-acquired' if starting before or during the two first days of hospitalization. In addition, infections were classified as 'health care associated' if patients had been hospitalized in the past 90 days, had undergone invasive procedures in a health care setting (*e.g.* injections, transfusion, urinary catheter placement) or had received chronic wound care at home as described by Friedman et al. [21].

Severity of co-morbidity was assessed using Charlson's Comorbidity Index (CCI) [22] as recently updated by Quan et al [23]. 'Shock' was defined as presenting with a mean arterial pressure of less than 60 mmHg despite adequate intravenous fluid resuscitation [16]. Hospital-associated outcome was assessed at the time of hospital discharge.

'Empirical antibiotic treatment' described the antibiotic(s) started upon patient admission before culture results were known, whereas 'directed antibiotic treatment' referred to treatment adapted to reported bacterial culture results. Appropriateness of antibiotic treatment was independently assessed by two infectious diseases physicians not involved in the clinical care of the patients studied (EV and WP), taking into account timing (*i.e.* ≤ 24 hours for initiation of empirical therapy), duration (*i.e.* ≥ 7 days for (directed) therapy), resistance patterns, clinical and pharmacokinetic data[24]. For ESBL-positive infections, all beta-lactam drugs were considered 'inappropriate' in a first assessment, as according to the local treatment guidelines [25]. Non-beta-lactam antibiotics were

considered appropriate according to the susceptibility pattern of the concerned isolate; in the case of fluoroquinolones and SMX-TMP only if the clinical presentation was non-severe. In a secondary analysis, we considered combinations of beta-lactam antibiotics with beta-lactamase inhibitors (bla/bli, *i.e.* amoxicillin-clavulanic acid in SHCH) as 'appropriate' in ESBL-positive infections provided the isolate displayed in vitro susceptibility

Statistical analysis

In descriptive analysis, frequencies and proportions were calculated for categorical variables, and the median and ranges for continuous variables. The Fisher's exact test was used for categorical variables. Relative risks (RRs) for acquisition of third generation cephalosporin resistance and for adverse outcomes mortality were calculated.

To identify independent predictors of ESBL acquisition, all variables with a p-value < 0.25 in univariate analysis were included in multivariate analysis. Subsequently, a backward selection process was performed, retaining those variables with a p-value < 0.05. In the multivariate analysis with adverse event as outcome, ESBL was the main exposure of interest. Empirical antibiotic treatment, the CCI and shock were a priori defined as potential confounders to be included in the model. In addition, those variables statistically significantly associated (p-value <0.05) with the outcome in univariate analysis were added. Patients referred early to another health facility were excluded from the adverse outcome analysis. Interactions were explored guided by bi-variate analysis and current knowledge. Co-linearity was assessed based on the calculation of variance inflation factors. Multivariate adjusted RRs were calculated using Poisson regression with robust error variance [26].

A number of sensitivity analyses were conducted to assess the robustness of the findings on adverse treatment outcome. This included the use of an alternative definition of appropriate empirical treatment (as defined above), inclusion of the information on directed treatment, and with the CCI treated as a continuous variable (after evaluation for the optimal functional form). Associations were considered statistically significant at p-values < 0.05. Data were analyzed using Stata version 11.1 (Stata Corp, College Station, Texas, USA) and Excel 2010 (Microsoft Corporation, Redmond, Washington, USA).

Ethical approval

Ethical approval was granted from the review boards at the Institute of Tropical Medicine, Antwerp, the University Hospital Antwerp and the National Ethics Committee on Health Research, Phnom Penh, Cambodia. Samples were taken as part of routine clinical care, not requiring prior informed

patient consent. Patients were identified with a unique hospital number while the anonymity status of the patients to any third party was preserved and guaranteed during and after the study. For the clinical and epidemiological data, no other data besides those noted in the routine medical files were used.

Results

Demographic and clinical data

In the study period 2007-2010, we identified 126 adult patients with an *E. coli* BSI (Figure 1). In ten patients (7.9%) the BSI episode was polymicrobial; concomitant pathogens included *Klebsiella pneumoniae* (n = 3), *Enterobacter* spp. (n = 2), *Enterococcus* spp. (n = 2), *Proteus vulgaris*, *Aeromonas* spp., *Histoplasma capsulatum* (n = 1 each). The patients' median age was 51 years (range 23-81), 62.7% were women. They came from 15 different provinces across Cambodia with the majority living in the greater area around the capital Phnom Penh (n = 38) and its neighboring provinces Kandal (n = 28), Kampong Cham (n = 16) and Kampong Speu (n = 7). About two thirds of all BSI (82/126, 65.1%) occurred during the wet season (May-October).

Several patients had one or more comorbidities, including diabetes mellitus (n = 27; 21.4%); human immune deficiency virus (HIV) infection (n = 23; 18.3%); chronic renal disease (n = 22; 17.5%) and chronic liver disease (n = 40; 31.0%). Twenty-two patients presented with signs of end-stage liver failure. Documented causes of chronic liver disease included alcoholism (n = 7), chronic viral hepatitis B (n = 3) and hepatitis C (n = 5). Etiologies of chronic renal disease included hepato-renal syndrome (n = 6), nephrotic syndrome, chronic hydronephrosis and vascular disease (n = 4 each). The median CCI was 1 (range 0-7).

Fifteen infections (11.9%) were considered nosocomial and an additional 24 (19.0 %) were healthcare-associated, while 87 infections (69.0%) were considered as community-acquired.

In 40 patients (31.7%) we noted prior antibiotic use in the past 3 months. In addition, another 49 patients (38.9%) reported having used 'an unknown drug' for febrile illness during that period. These prior antibiotic courses consisted of a single drug in 24 patients (18.9% of all patients) or a combination of 2 (12 patients; 9.5%), 3 (3 patients; 2.4%) or 4 (1 patient) antibiotics. The most commonly used antibiotic classes included penicillins (n = 17; 13.4%), fluoroquinolones (n = 13; 10.2%) and cephalosporins (n = 5; 3.9%).

A clinical focus of the *E. coli* BSI was presumptively identified in 102 (81.0%) episodes: urogenital infections (n = 48; 38.1%), spontaneous bacterial peritonitis (SBP, n = 16; 12.7%) and biliary tract

infections (n = 14; 11.1%) were the most frequent foci of infection. Other possible foci included other intra-abdominal (n = 9; 7.1%), respiratory tract infections (n = 10; 7.9%) and complicated skin and soft tissue infections (SSTI, n = 4; 3.2%). For 24 patients (19.0%) no clear primary foci could be found. Patients with SBP had more often high comorbidity rates (*i.e.* CCI ≥2; 14/16, 87.5%) as compared to patients with other diagnoses (48/110; 43.6%, p = 0.002).

Duration of illness could be assessed for 100 patients. Seventy-three patients (73.0%) patients with *E. coli* BSI presented with an acute febrile illness, 27 (27.0%) had a protracted disease course (≥ 3 weeks) prior to diagnosis. Thirty-two patients (25.4%) presented in septic shock, most commonly patients with urosepsis (15/48; 32.5%) and SBP (4/16; 25.0%).

Microbiological data and risk factors for acquisition of ESBL-producing E. coli BSI

Sixty-six (52.4%) episodes were caused by third generation cephalosporin-resistant (ceph3-R) *E. coli* and 60 (47.6%) by third generation cephalosporin-susceptible (ceph3-S) *E. coli* isolates, respectively. Out of these 66 ceph3R *E. coli*, 65 were ESBL-positive (of which 2 displayed co-carriage of plasmidic AmpC (pAmpC)); another isolate was pAmpC positive only (Figure 1). Predominant ESBL types were CTX-M-14 (n = 18) and CTX-M-15 (n = 35), 18 of the 65 (27.7 %) CTX-M-positive *E. coli* were of the ST 131-type. Further microbiological characterization of these isolates is reported elsewhere [15, 27].

Table 1 displays antibiotic resistance of the *E. coli* causing BSI, showing high level of (co)-resistance to commonly used antibiotics such as SMX-TMP, ciprofloxacin and gentamicin in both ESBL-positive and -negative *E. coli*.

In univariate analysis, significant risk factors for the acquisition of ESBL-positive infections were the prior use of antibiotics, including cephalosporins (Table 2). Likewise, patients with ESBL-positive *E. coli* BSI tended to be of younger age, to have higher rates of nosocomial infection and to have used more frequently penicillin and fluoroquinolone antibiotics prior to their BSI, but these associations did not reach statistical significance. In multivariate analysis, both prior antibiotic use (RR 1.46; 95% CI 1.03-2.09, p = 0.035) and use of cephalosporins (RR 1.59; 95% CI 1.23-2.05, p < 0.001) were found as independent risk factors for the acquisition of ESBL-positive infection.

Treatment, outcome and risk factors for mortality

Treatment and outcome data were available for 110 patients (54 patients with an ESBL-positive infection and 56 with a ESBL-negative infection). Empirical antibiotic treatment regimens included mainly ceftriaxone (n = 76), amoxicillin-clavulanic acid (n = 18) or ciprofloxacin (n = 8) with or without additional antibiotics *e.g.* metronidazole or aminoglycosides. Directed treatment schedules included meropenem (n = 19), amoxicillin-clavulanic acid (n = 21), ciprofloxacin (n = 9), ceftriaxone (n

= 4), amikacin (n = 3), sulphamethoxazole-trimethoprim (SMX-TMP, n = 1) and nitrofurantoin (n = 1) alone or in combination *e.g.* with metronidazole. In 50 patients the empirical therapy remained unchanged or therapy was discontinued because of referral or palliative care at home. Overall, 49 (44.5%) patients received inappropriate empirical therapy. Of those, 19 (17.3%) did also receive inappropriate directed therapy.

Patients with ESBL-positive infections received more frequently inappropriate empirical treatment as compared to patients with ESBL-negative infections (43/54, (79.6%) versus 6/56 (10.7%), p < 0.001), and the same trend was observed for combined inappropriate empirical and directed therapy (16 out of 54 (29.6%) ESBL-positive infections versus 3 of 56 (5.4%) ESBL-negative infections, p = 0.001).

Outcome was assessed after a median of 6 days (range 0-36 days) after the BSI. Thirty-two patients died (29.1%): 28 died in the hospital and 4 returned home for palliative treatment. In-hospital mortality occurred early (after a median of 2 days, range 0-30 days). Eight patients (7.3%) were referred to other hospitals and were excluded from further outcome-analysis. Out of the 70 (63.6%) surviving patients, six had a clinically or microbiologically confirmed recurrent infection within a median of 7 weeks (range 6-13 weeks).

Table 3 displays the univariate analysis of risk factors for mortality in all patients with *E. coli* BSI. Of note, a CCI ≥ 2 (RR 2.82 (1.34-5.92), p = 0.003), chronic liver disease (RR 1.91 (1.09-3.35), p = 0.042) and shock (RR 1.87 (1.07-3.25, p = 0.038), but not inappropriate empirical antibiotic treatment were significantly associated with mortality. Presence of ESBL-positive *E. coli* infection was more common among those who died (19/32; 59.4%) as compared to those who survived (31/70; 44.3%, p = 0.201) but this difference was not statistically significant. When repeated in the largest patient group with similar focus (*i.e.* patients with a urogenital focus), chronic liver disease remained the only significant risk factor for mortality (RR 7.11 (95% CI 2.63-19.20), p= 0.0003). In multivariate analysis (Table 4), a CCI ≥ 2 was the main independent risk factor (RR 2.75 (11.11-6.81, p = 0.028). For presence of shock and ESBL, we noted trends towards increased mortality risk. In all sensitivity analyses, the association between ESBL and adverse outcome remained statistically non-significant with adjusted RR's ranging from 1.66 to 1.85.

Discussion

In this study, we report the clinical characteristics and outcome in a cohort of adult patients with *E. coli* BSI in Cambodia. Urosepsis, spontaneous bacterial peritonitis and biliary tract infections were the most common infectious foci. The occurrence of ESBL-producing *E. coli* was very common, with

or without combined resistance to commonly available antibiotics such as ciprofloxacin, SMX-TMP and gentamicin. We identified the prior use of antibiotics, and particularly of cephalosporins as main risk factors for the acquisition of ESBL. This is in line with several other recent studies from Asia and Europe on community-acquired ESBL-positive *E. coli* bloodstream and other invasive infections [28-31]. Several authors have identified additional risk factors often connected to health care contact (*e.g.* recent hospitalization, instrumentation, recurrent infection)[5, 6, 32-34]; in our study we could only evidence a trend towards a higher risk of ESBL-acquisition in the nosocomial setting.

The overall mortality in our population -nearly 30%- was high and mainly related to patient-based variables, *i.e.* co-morbidity (particularly chronic liver disease) and severity of illness such as septic shock although we also observed a trend to higher mortality in ESBL-positive infections. Instead, we did not observe a significant influence of inappropriate empirical antibiotic treatment or of a specific clone such as *E. coli* ST 131. A similar risk factor pattern was also observed by several other authors[34-36].

The influence on outcome of inappropriate empirical antibiotic therapy in ESBL-positive *E. coli* BSI is subject of debate in the literature. Particularly in the earlier years of the ESBL-epidemic, the association between inappropriate empirical treatment and adverse outcome was described frequently [6, 28, 37-40], whereas more recently authors from several Asian and European settings mentioned the absence of this association [35, 36, 41-43]. Several interfering factors and methodological issues may explain these conflicting observations, including the shift from nosocomial to community-acquired infections, the regional presence of a particular clone and virulence factors, influence of the clinical focus (urinary tract infection (UTI) versus non-UTI infections), variable definitions of 'inappropriate therapy' and the studies' statistical power [24].

In the literature, mortality of *E. coli* BSI ranges between 10-30%, but most available data are from high or middle income settings. Mortality occurred early in the hospitalization, which may suggest that patients in our study had long referral and treatment delays and/or that the supportive care given was suboptimal.

Our study has several limitations. Given the retrospective and single-center nature of the study, the extent and level of available clinical data was limited. Also, a larger cohort of patients included over a longer time period would have improved the statistical power while studying risk factors and associations. Finally, only early outcome data were available whereas 30- and 90-day outcome assessments would provide more complete information on genuine mortality and relapses although longer assessment periods have the inherent risks of over-interpreting the attributable mortality [44].

In spite of these limitations, we believe this is the first study carried out in Cambodia which provides clinical and outcome data on a common type of invasive bacterial infection in resource-limited settings. In addition, the microbiological and clinical data were not only collected for research purposes but originated from a routine setting of a starting-up microbiology laboratory facility with attached surveillance and antibiotic stewardship activities.

While caution is warranted in extrapolating our findings to other health care settings in Cambodia and in the Southeast Asian region, our observations may shed new light on the management of severe bacterial infections in other resource-poor settings.

It is well known that the outcome of a patient with an invasive bacterial infection is the resultant of the complex interplay between the pathogen (including virulence factors, resistance genes, inoculum and infection focus) and the host (*i.e.* the genetic background, co-morbidity, clinical presentation, immune status and supportive care) [45]. With the emergence of antibiotic resistance and evidence on the role of appropriate antibiotic treatment in the severely ill [46], much emphasis in guidelines has been given to an early appropriate empirical antibiotic coverage. This has lead worldwide to the sharp increase in the usage of carbapenems and other broad spectrum antibiotics, which again has contributed to the worldwide emergence of even more difficult-to-treat resistance pathogens such as carbapenemase-producing *Enterobacteriaceae* (CPE) [47], leading to vicious circle of antibiotic use and resistance selection. However, our findings suggest that a 'blanket' broadening of the empirical antibiotic spectrum for all patients suspect of bloodstream infection will probably not be the most efficient intervention to reduce patient mortality.

Although assessment of the local quality and level of care for the critically ill was not the scope of the present study, the present findings highlight the importance of investments in patient-supportive interventions such as optimal sepsis care in all health care settings (*i.e.* early goal directed therapy within adapted sepsis bundles [48, 49], but also shortening referral delays, lowering (financial) hospital admission thresholds and adequate prevention and care of co-morbidities such as liver cirrhosis and diabetes mellitus. Standard treatment guidelines should also put more emphasis on the identification of those patients most at risk of dying and those who are most likely to benefit from empirical coverage with broad spectrum antibiotics. Whether the Charlson's Comorbidity Index or other scoring systems are most suitable in this low-resources setting should be further assessed.

Besides the improvement of patient outcomes, additional measures are urgently needed to curb the further selection and spread of ESBL under pressure of the widespread usage of ceftriaxone and

fluoroquinolones. These drugs have been already recognized for their higher potential of 'collateral damage' as compared to other antibiotics [50]. In Cambodia ceftriaxone is considered a cheap (around 1 US $ per 1 g- vial) and a practical drug for a myriad of conditions, while oral ciprofloxacin is popular for presumed typhoid fever and urinary tract infections. Quantitative monitoring of antibiotic usage patterns in Cambodia are lacking both in the community and in hospitals, but qualitative research revealed that drug dispensing by uncertified persons is very common [51] and antibiotics make out at least 60% of all prescription [52]. Like in many other LMIC, non-prescription use of antibiotics is probably very common [53]. Even less data are available on antibiotic use in the veterinary sector in Cambodia, although several local experts confirm that a broad range of oral and injectable antibiotics (including macrolides, cephalosporins, fluoroquinolones and possibly polymyxins) are used in an uncontrolled way as growth promoter and for curative purposes [54, 55]. This highlights the urgent need for more research and surveillance of antibiotic use in human and veterinary medicine in Cambodia and more broadly in the Southeast Asian region.

With regards to empirical treatment choices for presumed sepsis of urogenital and abdominal origin, we believe that the use of third generation cephalosporins should be discouraged and we suggest its replacement by amoxicillin-clavulanic acid in the absence of previous antibiotic exposure, but with the addition of amikacin in case of severe sepsis or of septic shock. For patients with recent prior antibiotic exposure, piperacillin-tazobactam with or without amikacin may constitute a suitable empirical choice. The use of carbapenems should be reserved for directed treatment in critically ill patients with proven ESBL-positive infections and should not be used in an empirical setting. In this study, adjustment of initially inappropriate empirical therapy based on antibiotic resistance data was performed in 30 of 49 patients. We assume this could have contributed to a favorable impact on patients' outcomes, and highlights the importance of building diagnostic microbiology capacity with a good and timely clinical interactivity.

Recent experiences of nationwide reduction of cephalosporin and fluoroquinolone use in other settings (*e.g.* the United Kingdom) are promising in terms of ESBL reduction [56]. However, caution is needed as increased usage of other antimicrobial classes (*e.g.* bla/bli antibiotics) may also exert an antibiotic pressure favouring the selection of other types of beta-lactamases such as OXA-48 carbapenemases or metallo beta-lactamases [56]. Therefore tight nationwide surveillance of invasive bacterial pathogens and its associated resistance mechanisms is warranted.

An additional caveat concerns patients' access to effective treatment. Amoxicillin-clavulanic acid is available in Cambodia nowadays in intravenous and oral formulations at costs around 1.75 US $ per vial of 1 g and 0.4 US$ per 625 mg tablet. Piperacillin-tazobactam is presently very scarcely available,

instead ampicillin-sulbactam is at a market price of 3.81 US $ per 1,5 g-vial, as is imipenem (15 US $ /500 mg vial). These drugs' costs may become quickly too high for people with an average monthly income of less than 100 US $. Affordability and availability may remain important thresholds to effective treatment in a country where hospital admissions often have dramatic financial consequences for patients and their families [57]. Therefore a sound national antibiotic policy should include measures to improve physical and economical access to life-saving drugs next to an enforced restriction of antibiotic use for doubtful indications in less ill patients.

Further research is needed to confirm these findings in a larger prospective study also including children and adult patients from rural as well as urban settings. Such a prospective, multi-centric study would allow to refine and locally validate scoring systems to identify those most at need of broad spectrum antibiotics. The necessity and safety of adding amikacin upfront in the most severely ill patients (*e.g.* those with severe liver disease) should also be studied in a randomized clinical trial.

Conclusion

In our study, *E. coli* BSI reflected a heterogeneous and comorbid patient group. Prior cephalosporin use constitutes a risk factor for acquisition of ESBL-positive *E. coli* BSI. Mortality was high and associated with severity of illness and comorbidities, suggesting an important role for the improvement of sepsis care.

For the empirical treatment of (presumed) sepsis of urogenital and abdominal origin, we suggest a policy to replace the use of ceftriaxone by bla/bli antibiotics such as amoxicillin-clavulanic acid and piperacillin-tazobactam with addition of amikacin in case of severe sepsis. Carbapenems should be reserved for directed treatment of critically ill patients with ESBL-positive *E. coli* BSI. This policy change would require however a strong surveillance system -both microbiological and clinical- and an ensured availability of these drugs.

Figures and tables

Figure 1. Flow chart of bloodstream episodes included in study.

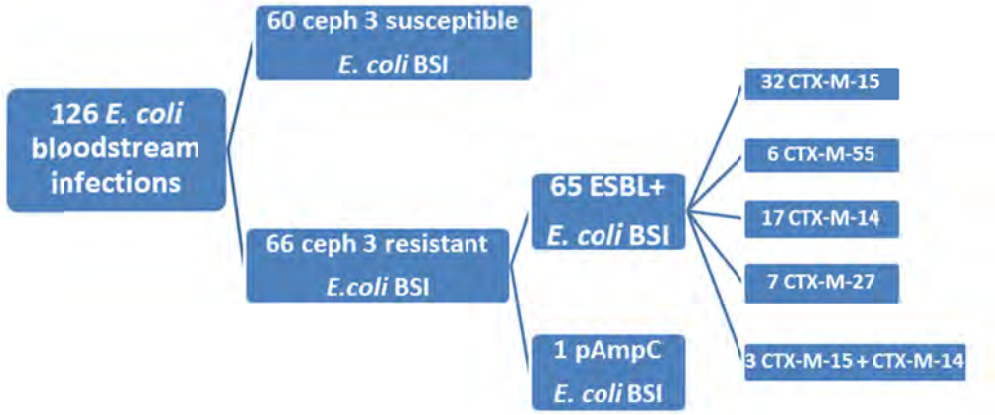

Table 1. Antibiotic resistance of ESBL-positive and ESBL-negative *E. coli* causing BSI in Cambodian adults (2007-2010).

	ESBL(+) *E. coli* (n = 65)		ESBL(-) *E. coli* (n = 61)	
	n	%	n	%
SMX-TMP	63	96.9	57	95.0
ciprofloxacin	62	95.4	20	33.3
gentamicin	53	81.5	17	28.3
meropenem	0	0.0	0	0.0
SMX-TMP + ciprofloxacin	60	92.3	19	31.7
SMX-TMP + ciprofloxacin + gentamicin	50	76.9	10	16.7
SMX-TMP + ciprofloxacin + gentamicin + amikacin	0	0.0	0	0.0

ESBL: extended spectrum beta-lactamase; SMX-TMP: sulphamethoxazole-trimethoprim; BSI: bloodstream infection

Table 2. Risk factors for acquisition of ESBL-positive *E. coli* BSI in Cambodian adults (univariate analysis)

	Patients with ESBL+ *E. coli* BSI (n = 65)		Patients with ESBL- *E. coli* BSI (n = 61)		RR	95% CI	*p*
	n	%	n	%			
female gender	40	61.5	39	63.9	0.95	0.67-1.35	0.854
age > 50	29	44.6	37	60.7	0.73	0.52-1.03	0.078
diabetes mellitus	12	18.5	15	24.6	0.83	0.52-1.31	0.515
HIV-infected	12	18.5	11	18.0	1.01	0.66-1.56	1.000
chronic liver disease	21	32.3	18	29.5	1.06	0.74-1.52	0.848
chronic renal disease	10	15.4	12	19.7	0.86	0.53-1.41	0.640
Charlson's comorbidity index ≥ 2	32	49.2	30	49.2	1.00	0.71-1.40	1.000
prior antibiotics (any)	27	41.5	13	21.3	**1.53**	**1.11-2.10**	**0.021**
prior use of penicillin-group AB	11	16.9	5	8.2	1.40	0.96-2.05	0.184
prior use of fluoroquinolone AB	9	13.8	4	6.6	1.40	0.93-2.10	0.244
prior use of cephalosporin AB	5	7.7	0	0.0	**2.02**	**1.69-2.41**	**0.058**
nosocomial infection	10	15.4	5	8.2	1.35	0.90-2.02	0.275
healthcare-associated infection	12	18.5	12	19.7	0.96	0.62-1.50	1.000
illness course ≥ 3 weeks	16	24.6	11	18.0	1.20	0.83-1.73	0.394
urogenital disease	28	43.1	20	32.8	1.23	0.88-1.72	0.273
SBP	7	10.8	9	14.8	0.83	0.46-1.49	0.596
biliary tract infection	6	9.2	8	13.1	0.81	0.43-1.53	0.576

ESBL: extended spectrum beta-lactamase; BSI: bloodstream infection; HIV: human immune deficiency virus; AB: antibiotic; SBP: spontaneous bacterial peritonitis; RR: relative risk; CI: confidence interval

Table 3. Risk factors for mortality of *E. coli* BSI in Cambodian adults (univariate analysis).

	died (n = 32)		survived (n = 70)				
	n	%	n	%	RR	95% CI	*p*
age > 50	16	50.0	39	55.7	0.85	0.48-1.52	0.671
female gender	21	65.6	44	62.9	1.09	0.59-2.00	0.828
ESBL-positive *E. coli*	19	59.4	31	44.3	1.52	0.84-2.74	0.201
CTX-M-15 carrying *E. coli*	10	31.3	18	25.7	1.20	0.65-2.21	0.635
E. coli ST 131	6	18.8	8	11.4	1.45	0.73-2.88	0.359
diabetes mellitus	7	21.9	17	24.3	0.91	0.45-1.84	1.000
HIV-infection	4	12.5	13	18.6	0.71	0.29-1.77	0.572
chronic liver disease	16	50.0	19	27.1	**1.91**	**1.09-3.35**	**0.042**
chronic renal disease	8	25.0	12	17.1	1.37	0.73-2.58	0.422
Charlson's comorbidity index ≥ 2	25	78.1	32	45.7	**2.82**	**1.34 - 5.92**	**0.003**
prolonged illness course	5	15.6	20	28.6	0.57	0.25-1.32	0.216
nosocomial infection	5	15.6	4	5.7	1.91	0.98-3.72	0.135
healthcare-associated infection	7	21.9	15	21.4	1.02	0.51-2.04	1.000
urogenital focus	11	34.4	33	47.1	0.69	0.37-1.28	0.283
SBP	6	18.8	7	10.0	1.58	0.81-3.09	0.336
hepatobiliary focus	3	9.4	10	14.3	0.71	0.25-2.00	0.750
shock	14	43.8	16	22.9	**1.87**	**1.07-3.25**	**0.038**
inappropriate empirical treatment *	15	46.9	29	41.4	1.16	0.66-2.06	0.669
inappropriate directed treatment *	6	18.8	14	20.0	0.95	0.45-1.99	1.000
inappropriate directed treatment **	5	15.6	13	18.6	0.86	0.39-1.94	0.787

**assessment with all beta-lactam antibiotics except meropenem considered inappropriate for ESBL-positive infection; ** assessment with amoxicillin-clavulanic acid considered appropriate for ESBL-infection if in vitro susceptible; ESBL: extended spectrum beta-lactamase; BSI: bloodstream infection; HIV: human immune deficiency virus; SBP: spontaneous bacterial peritonitis.*

RR: relative risk; CI: confidence interval

Table 4. Risk factors for mortality of *E. coli* BSI in Cambodian adult (multivariate analysis).

		Died		Unadjusted RR (95% CI) in univariate analysis	Adjusted RR (95% CI) in multivariate analysis	*p*
		n	%			
E. coli	ESBL+	19/50	38.0	1.52 (0.84-2.74)	1.93 (0.95-3.92)	0.071
	ESBL-	13/52	25.0			
chronic liver disease	Yes	16/35	45.7	1.91 (1.09-3.35)	1.09 (0.54-2.20)	0.810
	No	16/67	23.9			
Charlson's comorbidity index	≥ 2	25/57	43.9	2.82 (1.34-5.92)	**2.75 (1.11-6.81)**	**0.028**
	< 2	7/45	15.6			
shock	Yes	14/30	46.7	1.87 (1.07-3.25)	1.58 (0.91-2.77)	0.105
	No	18/72	25.0			
inappropriate empirical treatment	Yes	15/44	34.1	1.16 (0.66-2.06)	0.68 (0.31-1.48)	0.365
	No	17/58	29.3			

BSI: bloodstream infection.

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Chapter 6
Staphylococcus aureus bloodstream infections

This 40 year-old male was treated empirically with ceftriaxone for 10 days before a diagnosis was made of multifocal *Staphylococcus aureus* BSI with septic arthritis of metacarpo-phalangeal joint of the left index (shown on photograph) and of the left sterno-clavicular joint. The primary focus was a large gluteal abscess secondary to intramuscular injection of painkillers. He recovered after drainage of 300 milliliters of pus and a total of 6 weeks of cloxacillin therapy. Infection control measures at health care level could help prevent these cases.

Staphylococcus aureus bloodstream infections in Cambodian adults: molecular analysis, resistance patterns and outcome

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Abstract

Background

Staphylococcus aureus is a well-known cause of bloodstream infection (BSI) in high-income settings. In low- and middle income countries, epidemiological and resistance data of these infections are very scarce. We describe clinical, resistance and molecular aspects of *S. aureus* BSI among febrile adults in Phnom Penh, Cambodia.

Methods

Blood culture isolates and clinical data were collected from patients with BSI presenting at Sihanouk Hospital Centre of HOPE (July 2007-December 2010). Identification was performed by conventional methods. Antibiotic susceptibilities were assessed using disk diffusion and MicroScan® (Siemens Healthcare) according to CLSI guidelines. Molecular characterization included spa typing, Staphylococcal Chromosomal Cassette (SCC) mec typing, Multi Locus Sequence Typing (MLST) and screening for presence of Panton Valentin Leukocidin (PVL) and toxic shock syndrome toxin (TSST) encoding genes by PCR. Risk factors were assessed with Fisher's exact test using Stata version 10.2 (Stata Corp, College Station, Texas, USA).

Results

We observed 48 patients with *S. aureus* BSI with a median age of 40 years (range 16-71 y); 65.0% were male. Sixty percent of the infections were community-acquired, but 70.8% of these patients had a co-morbidity. Skin and soft tissue infections (SSTI) were the presumed focus in 54.2% infections . MRSA prevalence was 22.9% *i.e.* 13.3% among community-acquired infections and 38.9% health care-associated and nosocomial infections. Co-resistance to four non-beta-lactam antibiotics was present in 63.6% (7/11) of MRSA and 8.1% (3/37) of MSSA isolates. Risk factors for MRSA-positive infection were older age (RR 3.23, 1.19-8.80, p = 0.048) and superficial SSTI (RR 3.19, 1.09-9.36, p 0.036).

Mortality was 14.6%, main risk factor was age older than 50 years (RR 4.27, 1.14-15.9, p = 0.044). Molecular analysis revealed 24 different types, most commonly ST188/t189 (n = 9, of which 5 MRSA SCCmec IV), ST121/159 (n = 8; all MSSA and 7 PVL+), ST834/t1379 (n = 5; 4 were MRSA SCCmec IV and TSST+), and ST 398/t034 (n = 5; all MSSA and PVL+). Fourteen isolates (29.2%) carried PVL, mostly MSSA and related to SSTI.

Conclusion

S. aureus is a frequent cause of BSI in Cambodia, with a wide genetic variety and elevated rates of MRSA and multidrug resistance. This warrants the availability of effective antibiotics, nationwide surveillance and the implementation of infection control measures in the country.

Introduction

Invasive *Staphylococcus aureus* infections are a well-known cause of mortality and morbidity, both as community-acquired and hospital or health-care associated infections [1]. The worldwide emergence of resistance to methicillin and other antibiotic classes has further complicated the management and outcomes of these patients [2].

In-depth data on the epidemiology, clinical presentation and resistance patterns of invasive infections such as *S. aureus* bloodstream infection are mainly available from high-income settings where microbiological diagnosis and surveillance systems are available. In tropical low-resources settings, SAB are probably also very common but under-recognized [3] which may eventually lead to incomplete or inappropriate patient management.

A systematic review on bloodstream infections (BSI) in South and Southeast Asia reported that *S. aureus* was the second most common cause of BSI in adults and the fourth in children with an overall proportion of 8.5% of all BSI [4]; for most of the low- and middle income settings in this region, more information is required to fully understand the local epidemiology and clinical presentation of SAB.

In a recent blood culture-based prospective survey of adults attending Sihanouk Hospital Center of HOPE (SHCH), Phnom Penh, Cambodia between 2007 and 2010, *S. aureus* was found the most common Gram-positive pathogen and the fourth most frequent cause of BSI [5]. Here we report on the clinical presentation, resistance patterns, virulence factors, genetic relatedness and outcome of these adult SAB episodes.

Material and methods

Study setting

SHCH is a 40-bed non-government referral hospital in Phnom Penh, Cambodia, providing care for poor and chronically ill adults; microbiology facilities were installed in 2005. In 2007, a prospective study on the causes of BSI was initiated as part of antibiotic resistance surveillance and associated stewardship activities [5].

Microbiological work-up of isolates

Between 2007-2010, venous blood (2x10 ml) was drawn for culture with registration of demographic and clinical data from all adult patients presenting with signs of the Systemic Inflammatory Response Syndrome [6] as described in detail elsewhere [5]. All isolates from blood cultures grown in patients suspected of BSI between July 2007 and December 2010 were identified at SHCH using conventional

methods and disk diffusion (Neo-SensitabsTM, Rosco Diagnostica, Taastrup, Denmark) for in-vitro susceptibility testing. The isolates were stored locally at -70°C; all isolates identified at SHCH as *Staphylococcus aureus* were sent to ITM and the National Reference Centre for *S. aureus*, ULB-Hôpital Erasme, Brussels, Belgium for further analysis.

Confirmation of identification and susceptibility patterns was performed by disk diffusion (using Neo-Sensitabs™, Rosco Diagnostica, Taastrup, Denmark) and MicroScan (Combo 28, Siemens Healthcare Diagnostics, Deerfield, USA) with additional use of D-test for the detection of inducible clindamycin resistance [7]. Interpretative breakpoints were those defined by the Clinical and Laboratory Standards Institute (CLSI) [8], intermediately resistant isolates were considered as resistant. For the compilation of the resistance data, only the first isolate per BSI episode (defined as a 14-day period following the first day of BSI diagnosis) was considered.

Molecular characterization

Confirmation of species and methicillin resistance was performed with multiplex PCR for 16S rRNA, *nuc* gene and *mec*A respectively [9]. *Spa* typing was carried out as described earlier by Hallin and colleagues [10] and Staphylococcal Chromosomal Cassette (SCC)mec typing with multiplex PCR according to Kondo et al. [11], while Multi Locus Sequence Typing (MLST) was performed on one randomly selected isolate of each spa type [12]. We used Polymerase Chain Reaction (PCR) to detect the presence of Panton Valentin Leukocidin (PVL) and toxic shock syndrome toxin (TSST) encoding genes (*i.e. luk*S-*luk*F PV and *tst* respectively) [13]

Clinical data

Clinical and epidemiological data were retrieved from the laboratory request form and through retrospective review of the patients' files. Infections were considered 'nosocomial' (hospital onset) if they occurred more than two days after hospitalization and 'community-acquired' if starting before or during the two first days of hospitalization. In addition, infections were classified as 'healthcare-associated' if patients had been hospitalized in the past 90 days, had undergone invasive procedures in a health care setting (*e.g.* injections, transfusion, urinary catheter placement) or had received chronic wound care at home as described by Friedman et al. [14].

Severity of co-morbidity was assessed using the Charlson's Comorbidity Index (CCI) [15] as recently updated by Quan et al [16]. 'Superficial' skin and soft tissue infections (SSTI) included cellulitis, erysipelas, impetigo, infected wounds and other infected skin conditions; 'deep SSTI' included pyomyositis, other closed abscesses and necrotizing fasciitis.

'Shock' was defined as presenting with a mean arterial pressure of 60 mmHg or less despite adequate intravenous (IV) fluid resuscitation [6]. Outcome was assessed at the time of hospital discharge.

'Empirical antibiotic treatment' described the antibiotic(s) started upon patient admission before culture results were known, whereas 'directed antibiotic treatment' referred to treatment adapted to reported bacterial culture results. Antibiotic treatment was independently assessed for appropriateness by two infectious diseases physicians not involved in the clinical care of the patients studied (EV and WP), taking into account timing (*i.e.* ≤ 24 hours for initiation of empirical therapy), duration (*i.e.* ≥ 10 days for (directed) therapy), resistance patterns and clinical data [17].

'Recurrent infection' was defined as a new *S. aureus* BSI episode at least 14 days after the former isolate and after appropriate treatment of the patient. Recurrent infections were considered 'relapses' if the isolates' patterns if the molecular types were identical, otherwise they were considered 'new infections'.

Statistical analysis

In the descriptive analysis, frequencies and proportions were calculated for categorical variables, and the median and range for continuous variables. The Fisher's exact test was used to compare categorical variables. Relative risks (RRs) for the acquisition of methicillin resistance and for mortality were calculated. Associations were considered statistically significant at p-values < 0.05. Data were analyzed using Stata version 11.1 (Stata Corp, College Station, Texas, USA) and Excel 2010 (Microsoft Corporation, Redmond, Washington, USA).

Ethical considerations

Ethical approval was granted from the Institute of Tropical Medicine, Antwerp, the University Hospital Antwerp and the National Ethics Committee on Health Research, Phnom Penh, Cambodia. Samples were taken as part of routine clinical care, not requiring prior informed patient consent. Patients were identified with a unique hospital number. For the clinical and epidemiological data, no other data besides those noted in the patients' medical files were used.

Results

Demographic data

Between 2007 and 2010 we observed 51 *S. aureus* BSI episodes, representing 11.5 % isolates among 445 clinically significant pathogens recovered from blood cultures. These 51 episodes included 46

'first' (non-duplicate) episodes and five recurrences in five patients. Based on molecular typing (see below), two episodes were classified as reinfections (*i.e.* new 'first' episodes). The remaining three recurrences were found to be relapses (*i.e.* of the same strain) and these isolates were not considered for description of antibiotic resistance rates. Here we describe the characteristics of the total of 48 'first or new' (non-duplicate) episodes in 48 patients.

As shown in Figure 1, patients came from 13 different provinces in Cambodia (Figure 1), most commonly Phnom Penh area (n = 11), Kandal (n = 9) and Kampong Cham (n = 8). Sixty-five percent of patients (31/48) were male, with a median age of 40 years (range 16-71 y). Underlying chronic illness was present in 34 patients (70.8%) and included mainly chronic infection with the human immune deficiency (HIV)-virus (n = 17; 35.4%), diabetes mellitus (n = 5; 10.4%) and liver cirrhosis (n = 4; 8.3%).

Sixty-three percent of the *S. aureus* BSI (30/48) were community-acquired while 22.9% (11/48) and 14.6% (7/48) were considered healthcare-associated and nosocomial respectively. Twenty-six patients (54.2%) had a history of antibiotic use in the past 90 days. This included mostly beta-lactam antibiotics (n = 12), tuberculostatic drugs (n = 4) or sulphamethoxazole-trimethoprim (SMX-TMP, n = 3). Seven patients (14.6%) had taken more than one antibiotic prior to blood culture sampling.

Clinical presentation

The majority of patients (43/48; 89.6%) presented with acute fever and a single infection focus; in five patients (10.4%) multifocal disease was found. Skin and soft tissue infections (SSTI) were the most common underlying infection focus (n = 28; 58.3%). Of those, 18 were superficial SSTI and ten were deep SSTI. Other foci included respiratory tract (n = 3; 6.3%), osteo-articular infections (n = 2; 4.2%), peritonitis and urogenital infections (n = 1; 2.1% each). In 15 patients (31.3%) the infection focus was not clear. Ten patients (20.8%) presented with signs of septic shock and/or multiple organ failure.

Antibiotic resistance

Table 1 summarizes the phenotypic antibiotic resistance patterns in the 48 first isolates of *S. aureus* BSI. Eleven isolates (22.9%) were found methicillin resistant *Staphylococcus aureus* (MRSA); the MRSA prevalence among community-acquired infections was 13.3% (4/30) versus 38.9% (7/18) among health care-associated and nosocomial infections (p = 0.074). Resistance rates to the non-beta-lactam drugs clindamycin, fluoroquinolones, macrolides and tetracyclines were high in MSSA and very high in MRSA isolates. We observed combined resistance for clindamycin,

fluoroquinolones, macrolides, tetracyclines in seven of 11 MRSA (63.6%) and in three of 37 MSSA isolates (8.1%). Susceptibility to SMX-TMP and fusidic acid was somewhat better preserved, and no resistance to vancomycin was noted (MIC 90 was 1 µg/ml).

Table 2 displays the univariate analysis of risk factors for the acquisition of methicillin resistant *S. aureus* BSI. Significant risk factors for MRSA infection were age older than 50 years (RR 3.23 (95% CI 1.19-8.80), p = 0.048) and presenting with a superficial SSTI (RR 3.19 (95% CI 1.09-9.36), p = 0.036). A trend to increased risk for MRSA infections was noted for nosocomial and health care associated infections. Of note, deep SSTI were found exclusively in patients of the MSSA-group.

Treatment

Information on empirical and directed antibiotic treatment was available for 42 and 39 patients respectively. Most commonly used empirical treatment choices included cloxacillin (n = 14), ceftriaxone (n = 11), amoxicillin-clavulanic acid (n = 3) and lincomycin (n = 3) with or without other antibiotics. Overall, empirical antibiotic choices were considered appropriate for 32 of 42 patients (76.2%), *i.e.* 31 of 33 MSSA infections (93.9%) and one out of nine MRSA infections (11.1%). Directed antibiotic treatment was appropriate for 32 of 39 (82.1%) patients with treatment information available, including three out of nine MRSA and 29 of 30 (96.7%) MSSA infections. However, about a third (11/32; 34.3%) of those 32 'appropriate' directed treatment schedules covered an unnecessarily broad bacterial spectrum. For the 32 patients with appropriate directed treatment, complete information on antibiotic dosing and duration was available for 18 patients, which was compliant with the local SHCH standard treatment guidelines in 13 patients (72.2%).

Outcome

Outcome was assessed after a median of seven days (range 0-43) after blood cultures were taken. Fifteen percent of the patients died (7/48); mortality occurred after a median of two days (range 0-10). Six patients died during hospitalization and one returned home for palliative treatment. Three patients were referred to another hospital, and for another three the outcome was unknown. Of the 35 surviving patients, three had a subsequent relapsing infection (with the same *S. aureus* strain, after four, six and ten weeks respectively, Table 3) and another two patients had a reinfection (with a different *S. aureus* isolate, after 29 and 31 weeks respectively, see above). In one patient persisting fever was noted for a duration of three months.

As shown in Table 3, age older than 50 years was the single significant risk factor for mortality (RR

4.27 (1.14-15.94), p = 0.044). In addition, we noted a trend to higher mortality in patients presenting with multifocal disease (RR 2.96 (0.77-11.40), p = 0.188) or shock (RR 3.19 (0.88-11.5), p = 0.113) but we did not observe an association between mortality and methicillin resistance, bacterial virulence factors or inappropriate empirical therapy.

Genetic relatedness of isolates

As displayed in Table 4, 24 different spa-types were observed overall; the majority of them within the MSSA isolates (n = 22; 91.7%). Five dominant clones comprised more than half of the isolates (26/48; 54.2%): within MSSA these were ST121/t159 (n = 8; 8/9 PVL+), ST398/t034 (n = 5; all PVL+) and ST188/t189 (n = 4). Major clones within the MRSA group were ST188/t189-SCCmec IV (n = 5) and ST834/t1379-SCCmec IV (n = 4, all TSST+). All but 2 MRSA isolates carried SCCmec IV; one isolate carried SCCmec IX (ST9/t337), another SCCmec V (ST59/t437).

Of note, multidrug antibiotic resistance was highly prevalent in MRSA ST 188/t189 and ST 834/t1379, and to a lesser extent also in MSSA ST 121/t159 and ST 188. These clones were also highly prevalent in nosocomial or health care associated infections.

Fourteen (29.2%) isolates carried PVL, of which 13 (92.9%) were MSSA (particularly ST 121/t159), this was associated with high rates of SSTI (n = 10, 76.9%). In contrast, we observed presence of TSST in five isolates, of which four were MRSA ST 934/t1379. We did not observe septic shock in these patients but one patient died. Of note, three of five TSST-positive infections and four of 14 PVL-positive infections were health care-associated or nosocomial.

Discussion

In this study we found *S. aureus* to be a common cause of bloodstream infection in Cambodian adults; its most frequent clinical focus was SSTI. Nearly 25% of all *S. aureus* isolates were methicillin resistant, which was found more frequently in patients of older age, those with superficial SSTI and (not significantly) following healthcare contact. Both MRSA and MSSA displayed high levels of resistance against alternative, non-beta lactam antibiotics. In-hospital mortality was about 15%, and mainly associated with patient age and disease severity rather than antibiotic resistance, bacterial virulence or inappropriate empirical therapy. We observed a wide range of spa types -especially in MSSA isolates-, yet five dominant types comprised more than half of the isolates, including MSSA ST 121/159, MSSA ST 398/t034, MSSA/MRSA ST 188/t189 and MRSA ST398/t034. PVL-positive strains occurred in about 30% of isolates, predominantly in MSSA and were associated with severe SSTI.

About one fourth of all our patients was infected with a methicillin-resistant pathogen, mostly combined with multidrug resistance to non-beta lactam antibiotics. This resistance rate and pattern is comparable to those in prospective studies on SAB in Northeast Thailand (28%) [18] and Vietnam (19%) [19]. Higher MRSA prevalence (up to 50-70%) has been reported from East-Asian countries such as China, Japan and Taiwan [20, 21], although data may have been biased in several of these studies focusing on in-hospital patient populations. However, longstanding surveillance studies describe increasing resistance rates in the entire region [21], including the emergence of MRSA in the community with increasingly blurred borders between 'community-acquired' and 'health care associated' and nosocomial infections.

The high prevalence of PVL in community-acquired (MSSA) isolates has been a common finding in other Asian countries including Thailand (50%) [22], Indonesia (10%) [23], China (13%)[24] and also in sub-Saharan Africa *e.g.* Nigeria (40%) [25]. This is in contrast with industrialized countries where this virulence factor is found mainly in (community-acquired)-MRSA and rarely in invasive disease [26]; this is particularly the case in the US whereas in Europe a high proportion of PVL-positive isolates are methicillin-susceptible[26].

The molecular analysis of these isolates indicates the presence of a heterogeneous population, especially in MSSA. Some, but not all sequence types in our study matched with those described in Cambodian children (*i.e.* ST121/t159, ST834/t1379)[27], but the overall molecular pattern in this patient group was more diverse, which probably reflects differences in patient age, local environment or specific risk factors. The most common sequence type in our study (ST121/159) has spread throughout Asia, Europe and Africa [28, 29] and typically presents as MSSA carrying PVL -as is the case in our patient series-, although Chheng and co-workers described its presence as MRSA ST121/159 SCCmec V in 2 children from the Siem Reap area. Another dominant clone, MSSA ST188/t189 has been found also in neighboring countries *e.g.* Malaysia [30], Indonesia [31] and from BSI in China [32] whereas MRSA ST188/189 SCCmec V is also prevalent in Malaysia [33]. MRSA ST59/t437 is a common clone circulating in China, Taiwan, Hong Kong , Singapore and Vietnam [28].

Two clones have been associated in the literature with livestock, *i.e.* ST 398/t034 and ST9/t337. The former, frequently associated with MRSA in pigs and farmers in Europe and Asia [34], occurred in our study exclusively as MSSA, which has also been described earlier this year in China [32]. The combination of SCCmec IX in MRSA ST9/t337 reflects a novel type which was described in 2012 in Thai pigs and pork meat [35, 36] and earlier this year in an outpatient from Northeast Thailand [37]. To our knowledge, our study provides the first description of this sequence type in Cambodia and from an

invasive infection. The role of animals in these clones' spread in the Southeast Asian region merits in-depth study, as many of the region's citizens are (backyard) farmers for whom close contacts with animals is likely. The use of antibiotics in farm animals in Southeast Asia is largely unknown but probably high and largely unregulated [38, 39].

The mortality rate of 15% in our patient series was relatively low, as compared to 20-25% in SAB series from high-income countries [40], 44% in Thailand [18] and even >50 % in studies from India [41]. We assume the mortality rate is an underestimate due to the small sample size of our cohort and its short follow up time (precluding the observation of 30-day outcome), the referral hospital setting causing a bias towards 'survivors' and the dominance of the (presumptive) community-acquired SCCmec IV and V in MRSA-isolates, which have been associated with better outcomes as compared to the (presumptive) hospital-acquired SCC med I, II, III [42]. Nevertheless, within the same BSI surveillance study, we found much higher mortality data *e.g.* for BSI due to *Burkholderia pseudomallei* (52.7%) and *Escherichia coli* (26.7%) [5] suggesting that also pathogen-specific factors play a role.

Within our patient group, outcomes were associated with age and clinical presentation rather than resistance patterns, virulence factors or appropriateness of empirical therapy, which is still subject of pro-con debate [40] and function of several methodological issues [43, 44]. We think our findings represent a first snapshot on invasive *S. aureus* infection in Cambodian adults; these data should be interpreted cautiously but they warrant further confirmation in a prospective multicentric study in the country.

There were several limitations in our study. The particular setting of SHCH may have caused a bias in our study population towards the chronically ill, in particular to patients with HIV-infection, a known risk factor for invasive *S. aureus* infections. Likewise, the setting of a referral hospital in the capital did not allow the calculation of population-based incidence data of *S. aureus* BSI. In addition, the number of patients was rather small and the level of clinical details was not similar in all patients' files. Investigations for complications such as endocarditis and metastatic infections was not performed systematically but upon the discretion of the treating physician. Finally, the relatively short follow-up time for several patients may have caused underestimation of the number of relapses and adverse outcomes.

Yet, being the first data on *S. aureus* BSI in Cambodia and combining clinical, microbiological and molecular data, we think our findings shed more light on the epidemiology of invasive *S. aureus* infections in Cambodian adults. They also complement data on MRSA types causing SSTI in children

in Northwestern Cambodia [27], and epidemiological studies on *S. aureus* BSI from other Southeast Asian countries such as Thailand and Vietnam [18, 19].

Compared to the abundant Gram-negative resistance in Southeast Asia [5] and worldwide, Gram-positive resistance appears to be a lower burden. However, this may change quickly and successful, resistant clones may spread rapidly in health care facilities and in the community. The occurrence of methicillin resistance in a low-resources setting (such as Cambodia) has several potential implications at public health level.

First, unlike for many Gram-negative pathogens, sound evidence is available on the strong impact of infection control practices and rational antibiotic use towards the further selection and spread of MRSA in health care settings [45, 46]. Recent studies in several low- and middle income countries have shown that effective hand hygiene is also possible in these settings [47]. In 2011, Cambodia's Ministry of Health launched the country's first and much-needed plan for hospital infection control [48].

Second, the presence of a potential life-threatening and difficult-to-treat pathogen highlights the need for access to effective and affordable antibiotics of good quality. Cloxacillin is widely available and affordable in its oral shape, but IV cloxacillin stocks may be limited while for MRSA, glycopeptides such as vancomycin may not be commonly available in Cambodian public hospitals and cost about 10 US$ per 1 g-vial *i.e.* 20 US$ per day, which largely exceeds the budget of most Cambodian citizens and is associated with issues of toxicity, dosing and monitoring. This problem is under-documented and probably underestimated in many other low-resources settings [49]. While a safe and well-controlled provision of these drugs for use in proven MRSA cases should be ensured, we think it would be worthwhile to explore -within the limits of co-resistance patterns- alternative drugs for treatment of (methicillin resistant) *S. aureus* BSI, *e.g.* IV and oral SMX-TMP [50].

In addition to the provision of drugs , an effective and appropriate management of *S. aureus* BSI implies also adequate sepsis care, search and removal of primary and secondary infection foci , microbiological follow-up and correct dose and duration of the drugs of choice, of which we observed a clear 'learning curve' throughout the study years. We think these aspects should be included in the undergraduate and postgraduate education of health care workers, and they may be summarized and translated to the local setting into a local 'care bundle' such as was piloted recently in Thailand [51].

Finally, these first findings from a monocentric study highlight the need for the expansion of microbiological diagnostics in Cambodian clinical practice, and the set-up of genuine nationwide surveillance of bacterial resistance.

Conclusions

S. aureus causing BSI in Cambodia are diverse, and resistance is considerable, for which treatment options are very limited. Older age is a risk factor for acquisition of MRSA and for mortality. These findings warrant the expansion of the local microbiological capacity in the area for surveillance and improved patient management.

Figures and tables

Figure 1. Geographic distribution of *S. aureus* BSI cases.

Table 1. Antibiotic resistance in 48 'first episodes' of *S. aureus* BSI Cambodia (2007-2010)

Table 2. Comparison of patients with MSSA and MRSA BSI

Table 3. Risk factors for mortality in 42 *S. aureus* bloodstream infection with known outcome, Cambodia 2007-2010.

Table 4. Molecular analysis of 48 *S. aureus* isolates from BSI in Cambodian adults, 2007-2010

Figure 1. Geographical distribution of *Staphylococcus aureus* bloodstream infections , 2007-2010*

**For seven patients the place of residence was unknown*

Table 1. Antibiotic resistance in 48 'first episodes' of *S. aureus* BSI

Antibiotic	MRSA (n = 11)		MSSA (n = 37)	
	n	%	n	%
Penicillin	11	100.0	36	97.3
Clindamycin	11*	100.0	16**	43.2
Azithromycin	10	90.9	25	67.6
Tetracycline	9	81.8	17	45.9
Moxifloxacin	9	81.8	12	32.4
SMX-TMP	6	54.5	5	13.5
Fusidic acid	1	9.1	2	5.4
Vancomycin	0	0.0	0	0.0
Clindamycin + azithromycin	10	90.9	15	40.5
Clindamycin + azithromycin + tetracycline	8	72.7	8	21.6
Clindamycin + azithromycin + tetracycline + moxifloxacin	7	63.6	3	8.1
Clindamycin + azithromycin + tetracycline + moxifloxacin + SMX-TMP	4	36.4	1	2.7

**includes 4 isolates with inducible clindamycin resistance; **includes 7 isolates with inducible clindamycin resistance*

Table 2. Risk factors for the acquisition of methicillin resistance among 48 patients with *S. aureus* bloodstream infection.

	MRSA (n = 11)		MSSA (n = 37)				
	n	%	n	%	RR	95% CI	p*
Demographics							
male gender	7	63,6	24	64.9	0.96	0.33-2.82	1.000
age > 50	6	54,5	7	18.9	**3.23**	**1.19-8.80**	**0.048**
Comorbidity							
HIV-positive	4	36,4	13	35.1	1.04	0.36-3.06	1.000
diabetes	1	9,1	5	13.5	0.70	0.11-4.54	1.000
Charlson's comorbidity index ≥ 1	4	36,4	20	54,1	0.57	0.19-1.70	0.494
Contact with health care							
nosocomial or health care associated infection	7	63,6	11	29.7	2.92	1.00-8.59	0.074
prior antibiotic treatment	8	72,7	18	48.6	2.26	0.68-7.49	0.189
Infection focus							
SSTI (superficial)	7	63,6	10	27.0	**3.19**	**1.09-9.36**	**0.036**
SSTI (pyomyositis, closed abscess)	0	0,0	10	27.0	0.00	-	0.089

**Fisher exact test, differences with p < 0.05 were considered statistically significant. MRSA: methicillin resistance Staphylococcus aureus; MSSA: methicillin susceptible Staphylococcus aureus; HIV: human immune deficiency virus; SSTI: skin and soft tissue infection*

Table 3. Risk factors for mortality in 42 *S. aureus* bloodstream infection with known outcome, Cambodia 2007-2010

	died (n = 7)		survived (n = 35)				
	n	%	n	%	RR	95% CI	p*
Demographics							
male gender	6	85.7	22	62.9	3.00	0.40 - 22.56	0.392
age > 50 y	4	57.1	6	17.1	**4.27**	**1.14-15.94**	**0.044**
HIV-positivity	1	14.3	15	42.9	0.27	0.04-2.05	0.222
diabetes mellitus	0	0.0	5	14.3	0	-	0.569
Charlson's comorbidity index ≥ 1	2	28.6	19	54.3	0.40	0.09-1.84	0.410
Pathogen							
MRSA	1	14.3	8	22.9	0.61	0.08-4.45	1.000
PVL-positive	2	28.6	12	34.3	0.80	0.18-3.62	1.000
TSST-positive	1	14.3	4	11.4	1.23	0.18-8.25	1.000
Presumed focus							
superficial SSTI focus	1	14.3	15	42.9	0.27	0.04 - 2.05	0.222
deep SSTI focus	2	28.6	7	20.0	1.47	0.34-6.35	0.631
non-SSTI	1	14.3	4	11.4	1.23	0.18-8.25	1.000
Clinical presentation							
multifocal disease	2	28.6	3	8.6	2.96	0.77-11.40	0.188
shock	3	42.9	5	14.3	3.19	0.88-11.51	0.113
Management							
inappropriate empirical therapy	1	14.3	9	25.7	0.53	0.073-3.92	1.000

**Fisher 's exact test, differences with $p < 0.05$ were considered statistically significant. HIV: human immune deficiency virus; PVL: Panton Valentin Leukocidin; TSST: Toxic shock syndrome toxin; SSTI: skin and soft tissue infection*

Table 4. Molecular analysis of 48 *S. aureus* isolates from BSI in Cambodian adults, 2007-2010

n isolates	MLST CC	*spa* type	SCC mec*	R CliAz[1]	R CliAzTetMox[2]	PVL[3]	TSST[4]	male	HIV+	HCA[5]	SSTI/OA[6]	relapse	died
MSSA													
8	ST 121	t159	-	4	1	7	-	8	4	3	8	1	2
6	ST 188	t189 (4), t1350, t078	-	4	1	-	1	2	3	4	3	-	1
5	ST 398	t034	-	5	1	5	-	3	1	-	2	1	-
3	ST 1	t127, t177, t3963	-	-	-	-	-	3	1	1	1	-	-
2	ST 834	t3111, t1379	-	1	-	-	-	2	1	-	1	-	-
2	ND	t1038	-	-	-	-	-	1	1	-	-	-	-
2	ND	t359, t521	-	1	-	-	-	1	1	1	1	-	1
1	ND	t380	-	-	-	-	-	1	-	1	1	-	-
1	ND	t213	-	-	-	-	-	-	-	-	-	-	-
1	ND	t5078	-	-	-	-	-	-	1	-	1	-	-
1	ND	t550	-	-	-	-	-	-	-	-	-	-	-
1	ND	t570	-	-	-	-	-	-	-	1	1	-	-
1	ND	t701	-	-	-	-	-	-	-	-	-	-	1
1	ND	t878	-	-	-	-	-	-	-	-	1	-	1
1	ND	t903	-	-	-	1	-	1	-	-	-	-	-
1	ND	t1425	-	-	-	-	-	1	-	-	1	-	-
37			-	15	3	13	1	23	13	11	21	2	6
MRSA													
5	ST 188	t189	IV	5	4	-	-	4	1	2	3	1	1
4	ST 834	t1379	IV	4	3	-	4	2	2	3	2	-	-
1	ST 9	t337	IX	-	-	-	-	-	-	1	1	-	-
1	ST 59	t437	V	1	-	1	-	1	1	1	1	-	-
11				10	7	1	4	7	4	7	7	1	1

**for MRSA isolates only; 1 resistance to clindamycin and azithromycin, 2 resistance to clindamycin, azithromycin, tetracycline and moxifloxacin, 3 Panton Valentin Leukocidin, 4 toxic shock syndrom oxin, 5 health care associated or nosocomial, 6 skin, soft tissue and osteoarticular infections*

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Chapter 7
Streptococcus suis, another porcine surprise.

Besides *Salmonella* Choleraesuis and certain *S. aureus*, *Streptococcus suis* is yet another pig-related zoonotic pathogen causing invasive bacterial infections in Cambodian adults. People may have become infected through handling pigs or selling, buying and cooking infected pork meat. Close contacts with pigs in Cambodia may lead to many 'porcine surprises'…

Streptococcus suis invasive infections in Cambodian adults.

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Submitted for publication

Abstract

Background

Streptococcus suis (*S. suis*), an emerging zoonotic pathogen, has been described as an important cause of invasive bacterial infections in Asia. We report clinical, microbiological and molecular characteristics of 13 adult patients with invasive *S. suis* infection in Cambodia, identified during a bacterial surveillance study.

Methods

Blood and cerebrospinal fluid specimens were cultured as part of a microbiological prospective surveillance study. *S. suis* isolates were identified using conventional methods, API 20 Strep and serotyping and confirmed with DNA amplification of 16SrDNA and *cps2J*. Antibiotic susceptibilities were assessed using disk diffusion and Etest macromethod. Clonal relationships of all *S. suis* isolates were assessed by Pulsed Field Gel Electrophoresis (PFGE) and Multi Locus Sequence Typing. Screening for genes conferring antimicrobial resistance and potential virulence factors was performed with Polymerase Chain Reactions.

Results

Between 2007 and 2012 we identified 19 *S. suis* isolates from 13 adult patients (median age 50.5 years (33-85 y), 61.5% female). Patients presented with meningitis (n = 10, of which nine with concomitant bloodstream infection (BSI)), or primary BSI (n = 3). We observed no in-hospital mortality, but noted oto-vestibular sequelae in four patients.All isolates were found to be serotype 2 and belonged to sequence type (ST) 1. We identified nine different PFGE patterns, of which three closely related to southern Vietnam isolates' patterns. Six strains isolated from four patients (30.8%) were resistant to at least three drug classes: macrolides (erythromycin, azithromycin, tylosin), clindamycin and tetracycline, and one displayed additional resistance for sulphamethoxazole-trimethoprim and chloramphenicol. Macrolide resistance correlated with presence of *erm*(B), tetracycline resistance with *tet*(M) (19/19 isolates), and *tet*(O) (3/19 isolates). Virulence genes *mrp* and *sly* were found in all isolates and *epf* in 17/19 isolates.

Conclusion

This is the first detailed description of a case series of human infection in Cambodia to identify *Streptococcus suis* serotype 2 as one of the causative agents of meningitis and BSI in adult patients in Cambodia, requiring specific treatment. All isolates displayed combined antibiotic resistance but remained susceptible to penicillin. These findings warrant an expansion of diagnostic laboratories and surveillance of the food chain in Cambodia.

Background

Streptococcus suis is a zoonotic, Gram-positive pathogen which colonizes the nasopharyngeal tract in pigs and may cause invasive infection in piglets and pigs. Humans may acquire the pathogen occasionally by direct contact with infected pigs or their products (blood, meat, offal) or through ingestion of raw or undercooked high risk pork products, such as fresh blood pudding or intestinal organs [1]. *S. suis* infection in human most commonly presents as meningitis or bloodstream infection (BSI); other presentations such as endocarditis, arthritis and peritonitis have been described as well [2-4].

Human *S. suis* infections have been identified worldwide, but the pathogen is particularly emerging in Asia, [1]. The total number of reported human cases worldwide probably exceeds 1000; of those more than 80% occurred in the (South) Eastern Asian region [1]. In Vietnam, the pathogen was found to be the most important cause of bacterial meningitis [5, 6], whereas a large outbreak was described in Sichuan, China in 2005 [7] followed by several case series from Thailand, Hong Kong, Taiwan and Japan [8-11]. Although the majority of human *S. suis* infections worldwide is caused by bacteria of serotype 2, isolated cases due to *S. suis* of serotype 4, 14 and 16 have been reported [12, 13].

In Cambodia, a country of 14 million inhabitants and home to about 2 million pigs [14], occasional clinical cases of *S. suis* meningitis have been described [15] but data on its epidemiology, clinical presentation and outcome in human patients remain scarce. In July 2007, systematic surveillance of bacterial pathogens causing BSI and other invasive infections in febrile adults was started in Sihanouk Hospital Center of HOPE (SHCH), Phnom Penh, Cambodia [16]. Here we describe the epidemiological, clinical and microbiological characteristics of 13 human cases of *S. suis* infection as identified during these surveillance activities in SHCH.

Methods

Study setting, population

SHCH is a 40-bed non-government referral hospital in Phnom Penh, Cambodia. Together with its associated clinics it provides over 135,000 outpatient visits and about 1000 hospitalisations per year of adult patients from across Cambodia. From all patients presenting with signs of the Systemic Inflammatory Response Syndrome (SIRS) [17], venous blood (2 x 10 ml) was drawn for culture with registration of demographic and clinical data. Patients suspected of meningitis underwent lumbar

puncture at the discretion of the treating physician and according to the hospital's procedure [18]. Patients were identified with a unique hospital number.

Clinical data

Basic epidemiological and clinical data were collected at SHCH from the blood culture request form and by retrospective chart review using a pre-printed data collection form. Sequelae (including deafness and vestibular dysfunction) were diagnosed on clinical basis.

Sampling and culture of blood and cerebrospinal fluid (CSF)

Blood was cultured as described in Chapter 2; cerebrospinal fluid (CSF) was cultured on blood-, chocolate- and Sabouraud dextrose agar for 3, 3 and 6 days respectively.

As part of standard patient care, isolates were identified by conventional biochemical tests and assessed for antibiotic susceptibility by disk diffusion. Isolates were stored at -70°C on porous beads in cryopreservative (Microbank, Pro-Lab Diagnostics, Richmond Hill, Canada). Isolates identified as *Streptococcus suis* at SHCH, *i.e.* alpha-haemolytic and optochin resistant streptococci from blood and CSF, were retrieved from -70°C, checked for purity and further worked up at the Institute of Tropical Medicine (Antwerp, Belgium) and at the Oxford University Clinical Research Centre (OUCRU), Ho Chi Minh City, Vietnam. Confirmation of identification was performed by API 20 Strep (bioMérieux, Marcy l'Etoile, France), serotyping (Statens Serum Institute, Copenhagen, Denmark) and specific Polymerase Chain Reactions (PCR) amplifying 16SrDNA [19] and *cps* 2J genes [5] using extracted DNA (QIAGEN DNeasy, Germany) from those isolates.

Antibiotic resistance profiling

Susceptibility testing was performed for amoxicillin, ampicillin, cefepime, meropenem, imipenem, erythromycin, azithromycin, tetracycline, rifampicin, clindamycin, chloramphenicol, levofloxacin, ofloxacin, enrofloxacin, tylosin, tiamulin, sulphamethoxazole-trimethoprim and vancomycin by disk diffusion (Rosco, Taastrup, Denmark) and were later confirmed with Etest macromethod for penicillin, ceftriaxone, tetracycline, erythromycin, azithromycin, vancomycin, sulfamethoxazole - trimethoprim and chloramphenicol (bioMérieux, Marcy l'Etoile, France). Interpretative breakpoints were those described in CLSI M100-S23 [20] for meningitis by *Streptococcus pneumoniae* and viridans streptococci. In addition CLSI M31-A3 [21] was applied for enrofloxacin and Rosco Guidelines for Veterinary Practice [22] for tylosin and tiamulin resistance data. Intermediately resistant isolates were considered resistant. In patients with more than one *S. suis* isolate, only resistance data of the first isolate were displayed. *Staphylococcus aureus* (ATCC25923) and *Escherichia coli* (ATCC 25922) strains were used for quality control purposes. Isolates resistant to tetracycline and/or erythromycin were

further characterized by detection of the *erm*(A), *erm*(B), *mef*(A), *tet*(M), *tet*(O), *tet*(L), *tet*(K), *tet*(W) and mosaic *tet*(O/W/32/O) genes using multiplex PCR's as described previously [23].

Molecular typing

Pulsed Field Gel Electrophoresis (PFGE) with SmaI digestion was performed as described elsewhere [5] on all 19 isolates from Cambodian patients and on 6 additional isolates from Vietnamese patients, which were representative for different reported PFGE patterns [5] and 2 from healthy pigs from Vietnam for comparison of profiles. The genetic relatedness of PFGE band patterns were analyzed using Bionumerics software V5.1 (Applied Maths, Ghent, Belgium). Multi locus sequence typing (MLST) was performed as described previously [24].

Virulence factor profiling

The presence of genes encoding the putative virulence-associated factors muramidase released protein (*mrp*), extracellular protein factor (*epf/epf**), and suilysin (*sly*) was determined for all strains, using multiplex PCR as described [25]. *S. suis* serotype 2 strains BM407 and 89-1591 were used as positive and negative controls, respectively.

Ethical approval

Ethical approval was granted from the review boards at the Institute of Tropical Medicine (ITM), Antwerp, the University Hospital Antwerp and the National Ethical Committee, Phnom Penh, Cambodia respectively. In SHCH, sampling of blood and CSF for culture along with recording of clinical information are part of the standard clinical care for patients with a suspicion of invasive bacterial illness; a waiver for prior informed consent was obtained. Patients were identified with a unique hospital number while their anonymity status to any third party was preserved and guaranteed during and after the study. The *S. suis* isolates from Vietnamese patients which were included for comparison the PFGE patterns, have been reported earlier [5].

Results

Clinical and epidemiological findings

We identified 19 *S. suis* isolates from 13 infection episodes in 13 adult patients with a median age of 50.5 years (range 33-85 y); eight of them were women (61.5%). Within the study period, *S. suis* was the 10th cause of bloodstream infection in SHCH (n = 9/445; 2.0%), but it was the most frequent bacterial pathogen cultured from CSF *i.e.* 10 of 32 (31.3%) grown CSF samples during the study

period. Out of these 10 *S. suis* isolates in CSF, three were initially (mis)identified as '*Streptococcus pneumoniae*', two as '*Streptococcus species'* and one as 'viridans streptococcus'.

S. suis infections occurred sporadically throughout the study period, with a clustering of eight episodes between April and December 2010 (Figure 1). The majority of patients came from Kampong Cham province (n = 6) and other southeastern provinces bordering Vietnam (*i.e.* Prey Veng (n = 2), Kandal (n = 2) and Takeo (n = 1); two patients were living in the capital Phnom Penh (Figure 2).

Among 13 *S. suis* infected patients, five were (pig) farmers, for the other patients it was not possible to retrieve information regarding their occupation and other *S. suis* infection risk factors (Table 1). Most patients had no underlying pathologies except for one patient with a stable human immunodeficiency virus (HIV) infection (CD4-cell count of 570/µl), one pregnant woman and another patient with known alcoholism. All infections were community-acquired (*i.e.* without history of recent or ongoing hospitalization); all patients presented with acute febrile illness with (n = 10) or without (n = 3) meningeal signs with a median duration of three days (range 1-15 d) prior to hospital admission, details are listed in Table 1. Seven patients had taken an unknown drug (possibly an antibiotic) prior to admission.

Blood cultures were drawn in all 13 patients, lumbar puncture was performed in the 10 patients suspected meningitis *i.e.* all patients except patients 6, 7 and 10 (Table 1). Blood cultures grew *S. suis* in all patients except for the blood sample of patient 11. In the CSF samples taken, pleiocytosis with polymorph predominance was found (median 1300 WBC/µl (range 278-15.900 WBC/µl) and all 10 samples yielded growth of *S. suis*. Therefore a final diagnosis was made of culture-confirmed meningitis and BSI in nine patients, of culture-confirmed meningitis in one patient and of primary BSI in another three patients, as shown in Table 1. No evidence was found of another infection focus (*e.g.* endocarditis, septic arthritis) or the Streptococcal Toxic Shock Syndrome in these 13 patients.

The median duration of hospital stay was 10 days (range 1-24 d). With exclusion of the two patients (patients 4 and 10) who were referred early to another hospital, *S. suis* infected patients were treated for a median of 14 days (range 5-28 d). Patients were treated with various antibiotic regimens, the mainstay being ceftriaxone 2 g q12-24h with (or without) dexamethasone, according to the SHCH Standard treatment Guidelines [18] for the empirical treatment of meningitis and sepsis respectively (Table 1). In four patients a prolonged treatment of 21 days was given because of persistent symptoms. No in-hospital mortality was observed, but in four patients prolonged acoustic or vestibular problems were noted, which resolved in 2 patients after 1 month, but persisted in two

other until at least 22 and 46 days of follow-up respectively. Another patient had persisting low-grade fever after completion of the treatment course and was not seen at follow-up (Table 1).

Isolate typing, clonality and virulence

Results of DNA amplification for *S. suis* 16SrDNA and *cps2J* as well as of biochemical tests and serotyping confirmed that all isolates were *S. suis* serotype 2. MLST analysis of all isolates showed that they belonged to the sequence type 1 (ST 1) and clonal complex 1.

As shown in Figure 3, PFGE analysis of the nineteen isolates could be grouped into nine different PFGE-profiles fitting into four main clusters (A1, B2, D4, E5). The profiles within cluster A1 and profiles of isolates from patient 3 were closely related to older isolates from southern Vietnamese patients (BM 453 and BM 198 respectively [5, 24]). Isolates within Cambodian cluster A1 were also closely related to *S. suis* isolated from tonsils of healthy Vietnamese pigs (*i.e.* FX14 and FX22 [24], profiles not shown) whereas the profile of four isolates in Cambodian cluster E5 was identical to another southern Vietnamese isolate (BM 213). Of note, the two isolates (blood and CSF) from patient 2 displayed a slightly different PFGE patterns, which belong to two closely related clusters, but still remained highly related with percentage of similarity above 80%. Within PFGE-clusters, we did not observe clustering of cases from the same period or province.

All isolates carried the virulence factors *sly*, *epf* and *mrp* except for isolates from two patients lacking epf (patients 1 and 3). The absence of *epf* in these patients was not associated with a less severe illness course; instead patient 3 presented with transient deafness (Table 1).

Resistance data

All isolates were susceptible for penicillins (penicillin, ampicillin, amoxicillin), cephalosporins (ceftriaxone, cefepime), carbapenems (imipenem, meropenem) and fluoroquinolones (levofloxacin, ofloxacin) but wereoverall resistant to tetracycline (100%) while resistance for macrolides and clindamycin was seen in four (30.8%) and five (38.5%) isolates respectively (Table 2). One isolate (of patient 11) displayed combined resistance for tetracycline, macrolides, clindamycin, SMX-TMP and chloramphenicol (Figure 3). In addition, we observed intermediate resistance for chloramphenicol in 3 other isolates, and for the veterinary antibiotics enrofloxacin and tiamulin in one and all isolates repectively (Figure 3).

Tetracycline resistance phenotypes were all associated with the presence of *tet*(M) and five isolates (from three patients) additionally carried *tet*(O). The *tet*(L) gene was only present in one of the latter five isolates (Figure 3). In six isolates (from four patients) with macrolide resistance, the gene

encoding *erm*(B) was identified, these isolates displayed high level resistance to all tested macrolides (*i.e.* erythromycin, azithromycin, tylosin) and clindamycin as illustrated by the Minimal inhibitory Concentrations for 90% of isolates (MIC 90) of 256 µg/ml for erythromycin, azithromycin and clindamycin (Table 2).

Discussion

We present -to the best of our knowledge- the first detailed description of a case series of human *S. suis* infection in Cambodia. Patients were middle-aged or elderly and predominantly women, living in the Southeast region bordering southern Vietnam; they mainly presented as meningitis with concomitant bloodstream infection. Outcomes were favorable besides persisting oto-vestibular symptoms in about one third of the patients. There was overall susceptibility to penicillin and ceftriaxone, but high rates of tetracycline resistance and emerging high-level macrolide resistance were found. We observed a variety of (closely related) PFGE-patterns without clonal pattern, precluding a genuine outbreak. Yet several of these patterns were very closely related to PFGE-patterns of isolates isolated a few years earlier from patients in southern Vietnam.

Our study had several limitations. First, its retrospective character did not allow the collection of detailed information on exposure and risk factors. Next, we present only a small number of cases, precluding generalizations on clinical presentation or resistance patterns. Given the acute nature of the disease and its typical presentation in rural areas, we assume the patients attending SHCH represent only the tip of the iceberg; recent internet communications on other cases in Kampong Cham and Takeo provinces [26, 27] support this. In addition, the absence of in-depth clinical assessment including audiography and longer follow-up periods may have resulted in an underestimation of complications and sequelae. Future expansion of surveillance activities in rural provincial settings as well as the prospective collection of more detailed clinical and demographic information and the use of more refined screening methods such as *S. suis*-specific real-time PCR [5] may help to identify more cases and understand more of its epidemiology within Cambodia.

Yet these findings were derived from a starting-up microbiology laboratory and surveillance program embedded in a clinical setting. Health care settings with bacterial culture facilities have remained very scarce in Cambodia until date [28], but they may play an important role in the country as 'sentinel centre' and whistleblower. In addition, regional collaboration allowed a better insight in the epidemiology through comparison with isolates and findings from neighboring regions in southern

Vietnam. Finally, the addition of clinical findings to microbiological and molecular data was essential to understand better the local epidemiology of these pathogens.

The overall clinical presentation of the Cambodian patients with *S. suis* infection corresponds well with those as described in large patient series from Vietnam [5, 29], where meningitis with relatively low mortality rates is also the most common presentation. We observed also broad correspondences with isolates from Vietnam in terms of serotype, sequence type, virulence factors and resistance patterns. Upon MLST, all our isolates belonged to ST1, a common sequence type which has been associated with meningitis and sepsis in human infection but with relatively low mortality rates (< 10%) [30], which contrasts with the very high case fatality rates (*i.e.* 64%) observed in infections caused by *S. suis* of ST7 during the 2005 outbreak in Sichuan, China [7, 31]. All but 2 patients have isolates carrying *mrp*, *ef* and suilysin, which are typical virulence factors for Eurasian ST1 strains [32]. We observed deafness and /or vestibular damage in 4 out of 13 patients (30.8%), which is relatively low as compared to 50-65% in other case series. Due to the limitations of our retrospective study, we may have missed milder cases; in addition the administration of dexamethasone to five patients with meningitis may have had a beneficial effect on the prevention of deafness and other long-term sequelae [1]. Where used at the discretion of the local clinician in SHCH between 2007 and 2011, the addition of a 5-day course of dexamethasone 8-10 mg IV q8h to the empirical antibiotic treatment was formally included in the first issue of SHCH's Standard Treatment Guidelines (September 2011) for the treatment of all patients with presumed bacterial meningitis except for those with known or suspected HIV status or tuberculosis. This was based on evidence form international [33] and regional [34] research. For *S. suis* meningitis, the beneficial effect of corticosteroid therapy added to empirical antibiotic treatment has been illustrated also from in-vitro [35] as well as in patient studies [5]. Of note, we did not observe a clear correlation between presence of the ef virulence factor and the occurrence of deafness or other sequelae.

Correspondences with southern Vietnam isolates were also found within the PFGE profiles. In particular profiles in the Cambodian cluster E5 were similar to those described elsewhere within the large cluster D from southern Vietnam isolates [5] and isolates described earlier within group III from northern Vietnam [29]. In addition, the Cambodian PFGE profile F6 was shown to be closely related to a large cluster A form southern Vietnam isolates [5], and Cambodian isolates of cluster A1 displayed high similarity with *S. suis* isolates from human and pig origin in Vietnam [24].

We observed also a particular difference: the majority of our patients were women. This contrasts with the overwhelming majority of male patients with *S. suis* infection in Vietnam, China and

Thailand, which has been attributed to gender-related occupational or behavioral risk factors such as slaughtering or eating traditional at-risk dishes [36]. Possibly women in our case series got infected through handling pigs or raw meat at markets or while preparing meals rather than eating dishes containing raw blood or pork products, which are not a culinary habit in the affected provinces unlike in several areas in Vietnam [36]. As it was not possible to re-contact patients for in-depth questioning regarding recent slaughtering or other known at risk activities, a better understanding of risk factors in Cambodia will require detailed, prospective questioning of future patients and control groups.

Our findings may have several implications for (public) health care in Cambodia and other low and middle income countries (LMIC) where *S. suis* occurs. Awareness of *S. suis*-related disease among local health care professionals and the general public including pig- or pork-exposed individuals may help to speed up the diagnosis and initiation of correct treatment. Training and support at local microbiology laboratories may help to prevent misidentification as 'pneumococci', 'viridans streptococci' [1]. In countries where *S. suis* appears to be endemic such as Cambodia, its needs also to be taken into account in the redaction and revision of treatment national guidelines, as *S. suis* treatment typically requires prolonged antibiotic treatment (up to 3 weeks) with the addition of corticosteroids for prevention of oto-vestibular sequelae as was illustrated in high-burden settings such as Vietnam and China [5, 8]. According to the resistance patterns of circulating isolates , (high dose) ceftriaxone appears an effective antibiotic choice for empirical therapy in patients with presumed bacterial meningitis, while non-meningitis presentations are likely to be covered by empirical use of amoxicillin or amoxicillin-clavulanic acid. Patients with *S. suis* meningitis are at risk for prolonged or relapsing infection with or without persisting neurological sequelae, and require careful assessment and follow-up. Specialized otologic services including audiometry may not be widely available in LMIC, therefore the World Health Organization issued freely-available training modules for 'Primary Ear and hearing care' [37] which includes extensive clinical guidance for diagnosis and rehabilitation of hearing disorders in LMIC. Mobile-phone-based audiologic monitoring may be a promising tool for affordable diagnosis of hearing disorders in LMIC [38].

Next, *S. suis* infections are, given their zoonotic nature closely related to safety of the food chain. The very close correlations between the *S. suis* isolates found in Cambodia and those from southern Vietnam are remarkable, given the genetic heterogeneity described in this pathogen. The apparent clustering of *S. suis* infections in 2010 coincides with communications from the Cambodian Ministry of Health on an outbreak of Porcine Reproductive and Respiratory Syndrome (PRRS) among pigs in

several provinces including Prey Veng, Kampong Cham, Svay Rieng, Takeo, Kandal and Kampot provinces [39]. PRRS may cause a chronic viral infection in pigs and makes them vulnerable for invasive bacterial infections such as *S. suis* [40]. In the same period (June-August 2010) increased numbers of porcine and human *S. suis* infections associated pig outbreaks with PRRS were described in southern Vietnam [41]. These findings warrant tightened surveillance of outbreak-prone pathogens in livestock and at wet markets.

Finally, zoonotic pathogens such as *S. suis* act also as sentinels for emerging resistance and associated intense use of antibiotics in livestock. Although macrolide antibiotics are not treatment of choice for invasive *S. suis* infections, the *erm*(B)-mediated azithromycin resistance we observed is of concern. Also in *Salmonella* Choleraesuis, another pig-related zoonotic pathogen prevalent in Cambodia, we described surprisingly high rates of azithromycin resistance [42]. A possible common cause could be the widespread use of tylosin, a 16-ring macrolide which is worldwide used liberally for a wide range of veterinary indications [43]. The product is cheap and probably also widely used in livestock without prescription in Vietnam [44] and Cambodia [45, 46] although solid data on antibiotic use in livestock are hard to find. Tight surveillance and restriction of antibiotic use in animals in the Southeast Asian region are urgently needed.

Conclusion

We conclude that *Streptococcus suis* is a common pathogen causing severe infections in Cambodia. In this case series we observed strong links with *S. suis* strains circulating in southern Vietnam. More research and surveillance on its extent, risk factors and emerging antibiotic resistance are needed**.**

Figures and tables

Figure 1: Temporal clustering of thirteen *S. suis* infected human cases, 2007-2012

Figure 2: Geographical distribution of thirteen human cases infected with *S. suis* in Cambodia

Figure 3. PFGE with SmaI digestion of *S. suis* strains isolated from humans and pigs in Cambodia and Vietnam.

A total of twenty seven *S. suis* isolates including nineteen (from thirteen Cambodian patients, 2007-2012) and 6 comparator strains isolated from adult patients [5]. A dendrogram was generated by Dice analysis (optimization, 0.5%; band tolerance, 1%) and cluster analysis with unweighted pair group method with arithmetic mean, using Bionumerics software 5.1 (Applied Maths). Bars indicate 95% CIs.. BM indicates isolates from Vietnamese bacterial meningitis patients. Three provinces Ho Chi Minh City (HCMC), Long An and Ben Tre are in Viet Nam; others are Cambodian provinces. Antibiotic names between brackets refers to intermediate resistance. Tet: tetracycline, Tyl: tylosin, Tiam: tiamulin, Ery: erythromycin, Azi: azithromycin, Clin: clindamycin, Chlor: chlamphenicol, Enrof: enrofloxacin, SMX: sulphamethoxazole-trimethoprim

Table 1. Clinical and demographic characteristics in thirteen patients with invasive *Streptococcus suis* infection attending Sihanouk Hospital Centre of HOPE, 2007-2012

Table 2: Minimal inhibitory concentrations of 13 non-duplicate *S. suis* isolates from patients attending SHCH, Cambodia (2007-2012)

Figure 1. Temporal clustering of 13 *S. suis* cases attending SHCH over study period

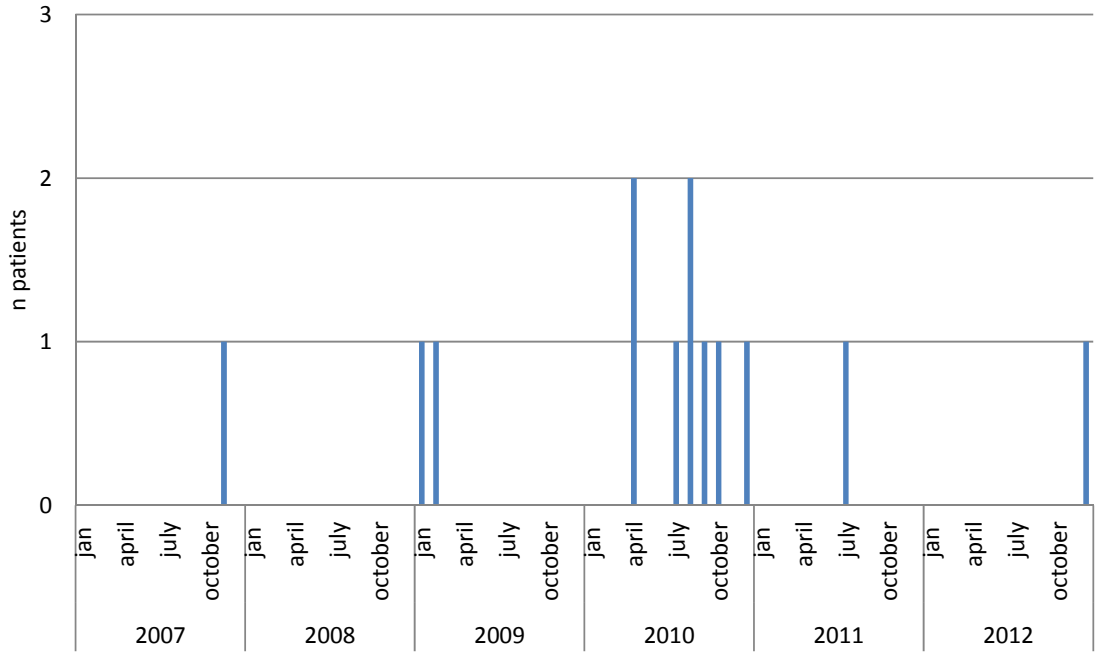
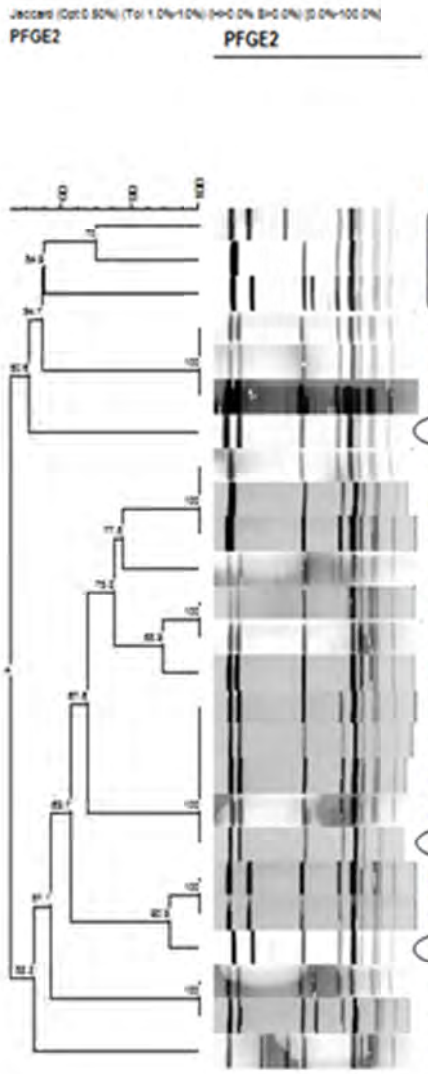


Figure 2. Geographical distribution of 13 *S. suis* patients from Cambodia and of 6 comparator cases from Southern Vietnam

Figure 3. Dendrogram of 19 *S. suis* isolates of 13 Cambodian patients (2007-2012) and 6 comparator Soutern Vietnamese isolates (1998-2004).



Key	Cluster/ pattern	Year	Patient	Province	Source	Phenotypic resistance	Resistance genes	Virulence genes
BM329	VN-C-7	2003	VN	HCMC	blood	Tet, Ery, Azi	*tet*(O), *erm*(B)	mrp, epf, sly
BM99	VN-F-16	1998	VN	HCMC	CSF	Tet	*tet*(M)	mrp, epf*, sly
BM91	VN-B-3	1998	VN	HCMC	CSF	Tet	*tet*(M)	mrp, epf*, sly
2234	A-1	2010	6	Takeo	blood	Tet, (Tiam)	*tet*(M)	mrp, epf, sly
2390		2010	9	P.Veng	CSF	Tet, (Tiam)	*tet*(M)	mrp, epf, sly
2391		2010	9	P.Veng	blood	Tet, (Tyl, Tiam)	*tet*(M)	mrp, epf, sly
BM453	VN-E-14	2004	VN	Ben Tre	CSF	Tet	*tet*(M)	mrp, epf, sly
2287	B-2	2010	8	K. Cham	CSF	Tet, (Tiam)	*tet*(M)	mrp, epf, sly
2307		2010	8	K. Cham	blood	Tet, (Tyl, Tiam)	*tet*(M)	mrp, epf, sly
580		2009	2	P Penh	blood	Tet, (Tyl, Tiam)	*tet*(M)	mrp, epf, sly
4895	C-3	2012	13	Kandal	blood	Tet, (Tiam)	*tet*(M)	mrp, epf, sly
105	D-4	2007	1	Kandal	CSF	Tet, (Tyl, Tiam)*	*tet*(M)	mrp, sly
2528		2010	10	K. Cham	blood	Tet, (Tiam)	*tet*(M)	mrp, epf, sly
579		2009	2	P Penh	CSF	Tet, (Tyl, Tiam)	*tet*(M)	mrp, epf, sly
1780	E-5	2010	4	P Penh	CSF	Tet, Clin (Enrof, Tyl, Tiam)	*tet*(M)	mrp, epf, sly
1786		2010	4	P Penh	blood	Tet, (Tyl, Tiam)	*tet*(M)	mrp, epf, sly
2290		2010	7	K. Cham	blood	Tet (Chlor, Tyl, Tiam)	*tet*(M)	mrp, epf, sly
2869		2010	11	P.Veng	CSF	Tet, Ery, Azi, Clin, Chlor, SMX, Tyl, (Tiam)	*tet*(M), (O), (L); *erm*(B)	mrp, epf, sly
BM213	(VN-D-11)	2000	VN	Long An	CSF	Tet	*tet*(M)	mrp, epf, sly
656	F-6	2009	3	K. Cham	CSF	Tet, Ery, Azi, Clin, Tyl, (Chlor, Tiam)	*tet*(M), (O); *erm*(B)	mrp, sly
657		2009	3	K. Cham	blood	Tet, Ery, Azi, Clin, Tyl, (Tiam)	*tet*(M), (O); *erm*(B)	mrp, sly
BM198	(VN-A-1)	2000	VN	HCMC	CSF	Tet, Ery, Azi	*tet*(O), *erm*(B)	mrp, epf*, sly
1855	G-7	2010	5	K. Cham	CSF	Tet, Ery, Azi, Clin, Tyl, (Chlor, Tiam)	*tet*(M), (O); *erm*(B)	mrp, epf, sly
1862		2010	5	K. Cham	blood	Tet, Ery, Azi, Clin, Tyl, (Tiam)	*tet*(M), (O); *erm*(B)	mrp, epf, sly
3543	H-8	2011	12	K. Cham	blood	Tet, Ery, Azi, Clin, Tyl, (Tiam)	*tet*(M); *erm*(B)	mrp, epf, sly

Table 1. Overview of clinical and demographic characteristics in 13 patients with invasive *Streptococcus suis* infection attending SHCH, 2007-2012.

Pat	Age	Gender	Province	Pig exposure	Co-morbidity	Symptoms	Culture date	Culture(s) positive	Diagnosis	Treatment	Outcome on discharge
1	60	M	Kandal	no information	no data	ND	28/11/07	blood*+, CSF +	meningitis, BSI	unknown	resolved
2	85	F	Phnom Penh	no information	none	fever 1d, falling, vomiting, stiff neck, lung crackles	11/01/09	blood+, CSF+	meningitis, BSI	ceftriaxone 2 g q12h x 21 d	resolved
3	51	F	Kampong Cham	farmer	none	fever 15 d, headache, back pain, stiff neck	27/02/09	blood+, CSF+	meningitis, BSI	ceftriaxone 2 g q12h x 14 d, ampicillin 2 g q4h x 3 d, dexamethasone 8 mg q8h x 3 d	transient deafness 1 month
4	78	F	Phnom Penh	no information	none	meningeal signs, dysuria	11/04/10	blood+, CSF+	meningitis, BSI	ceftriaxone 2 g once	referred day 1
5	57	F	Kampong Cham	farmer	none	fever 2 d, headache, confusion, vomiting	28/04/10	blood+, CSF+	meningitis, BSI	vancomycin 1 g once, ceftriaxone 2 g q24h x 7 d, cloxa-ampicillin 2 g q4h x 21 d, dexamethasone 8 mg q8h x 3 d	persisting low grade fever
6	63	M	Takeo	no information	none	fever 7 d, cough, headache, weigth loss	28/07/10	blood+	BSI	ceftriaxone 2 g q24h x 7 d	resolved
7	39	F	Kampong Cham	no information	HIV+	fever, SSTI	10/08/10	blood+	BSI	ceftriaxone 2 g q24h x 5 d, then amoxicillin 500 mg q8h	resolved
8	47	F	Kampong Cham	farmer	none	fever 2 d, headache, confusion, agitation	11/08/10	blood +, CSF+	meningitis, BSI	ceftriaxone 2 g q12h x 21 d, then benzathin penicillin 2.4 MU/w x 3 w, dexamethasone 8 mg q8h x 3 d	persisting deafness, headache > 3 weeks
9	50	M	Prey Veng	no information	none	fever	6/9/10	blood +, CSF+	meningitis, BSI	ceftriaxone 2 g q24h x 5 d (?)	resolved
10	33	F	Kampong Cham	no information	none	acute fever, respiratory symptoms	18/10/10	blood+	BSI	ceftriaxone 2 g q24h x 4 d, metronidazole 500 mg q8h x 4 d	referred day 4
11	45	F	Prey Veng	farmer	pregnant	fever 4 d, severe headache, vomiting, stupor 2 d, shock	30/12/10	blood -, CSF+	meningitis	ceftriaxone 2 g q12h x 14 d, ampicillin 2 g q4h x 7 d	persisting deafness and headache > 6 weeks
12	51	M	Kampong Cham	pig farmer	none	fever 4 d, severe headache, neck stiffness	28/07/11	blood +, CSF*+	meningitis, BSI	ceftriaxone 2 g q12h x 12 d, dexamethasone 4 mg q8h x 5 d	transient dizziness 1 month
13	39	M	Kandal	no information	alcoholism	fever 2 d , severe headache, vomiting, confusion, septic shock	21/12/12	blood +, CSF*+	meningitis, BSI	ceftriaxone 2 g q24h x 23 d, penicillin G 4 MU q4h x 7 d, dexamethasone 8 mg q8h x 5 d	resolved

* isolate not available for further analysis; CSF: cerebrospinal fluid; BSI: bloodstream infection; MU: million units; SSTI: skin and soft tissue infection

Table 2. Minimal inhibitory concentrations of 13 first *S. suis* isolates from patients attending SHCH, Cambodia (2007-2012)

	Resistance breakpoint (µg/ml)	MIC range (µg/ml)	MIC 50 (µg/ml)	MIC 90 (µg/ml)	Resistant n (%)
penicillin	≥ 0.12	0.032 - 0.047	0.047	0.047	0 (0.0)
ceftriaxone	≥ 2	0.023 - 0.125	0.064	0.125	0 (0.0)
tetracycline	≥ 4	12 - 256	32	256	13 (100)
SMX-TMP	≥ 4	0.023 - 256	0.064	0.19	1 (7.7)
erythromycin	≥ 1	0.032 - 256	0.064	256	4 (30.8)
azithromycin	≥ 2	0.125 - 256	0.25	256	4 (30.8)
clindamycin	≥ 1	0.064 - 256	0.125	256	5 (38.5)
chloramphenicol	≥ 8	3 - 32	4	6	1 (7.7)
vancomycin	> 1	0.38 - 0.75	0.5	0.75	0 (0.0)

MIC: minimal inhibitory concentration; SMX-TMP: sulphamethoxazole-trimethoprim

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Chapter 8.

General discussion and conclusion

Bacterial multidrug resistance is a genuine challenge for patient care, from the correct diagnosis over availability of effective drugs to the ethics of infection control measures. This is even more true in low-resources settings where resistance rates are high and means are scarce.

8.1. Synopsis of research findings

As shown in Chapter 1, invasive bacterial infections are a worldwide major cause of severe morbidity and mortality, particularly in the case of BSI. The fast global spread of antibiotic resistance has radically reduced choices in antibiotic treatment, which is one of the cornerstones of a successful management of severely ill patients. The prevalence of invasive bacterial infections in LMIC is not well known but is estimated to be higher than in high-income settings such as Europe and the US. In many LMIC, large knowledge gaps remain regarding the local spectrum and resistance patterns of pathogens causing invasive bacterial infections, which is in turn reflected in outdated or inappropriate treatment guidelines for the management of febrile patients[1]. The studies presented in this thesis describe the main pathogens of invasive bacterial infections in adults attending a referral NGO hospital in Phnom Penh, Cambodia and present details of their clinical presentation and outcome, resistance patterns and mechanisms and genetic relatedness to circulating strains in the Southeast Asian region.

Spectrum of pathogens: bio-diversity fitting within the region

In Chapter 2 we noted that the majority of the pathogens causing community-acquired BSI were Gram-negative pathogens. *Escherichia coli*, but also *Klebsiella pneumoniae*, *Salmonella enterica* and non-fermentative rods such as *Burkholderia pseudomallei* -the etiological agent of melioidosis-, were predominant among the causative agents of BSI, while *Staphylococcus aureus* was the single most prevalent Gram-positive pathogen. In addition, *Streptococcus suis* was occasionally found among patients with BSI and meningitis. In contrast, we observed very few infections caused by *Streptococcus pneumoniae*. These data reflect the biodiversity of pathogenic bacteria in Cambodia, where environmental and zoonotic pathogens (*e.g. B. pseudomallei*, *Salmonella* Choleraesuis, *S. suis*) appear to be superimposed to cosmopolitic pathogens such as *E. coli* and *S. aureus*.

This predominance of Gram-negative pathogens has been reported frequently from other community-acquired BSI studies in LMIC in Southeast Asia and other regions[2-5] although considerable differences exist in the etiology of BSI between adult and pediatric populations, and between BSI studies from sub Saharan Africa and Southeast Asia. In their systematic review of 17 BSI studies from South and Southeast Asia, Deen and colleagues noted Gram-negative causal pathogens in 77.4% of adult patients (mainly *Salmonella enterica* and *Enterobacteriaceae*) but not so among children, where *S. pneumoniae* and *S. aureus* also played an important role [6]. In a pediatric BSI study from Siem Reap in Northeast Cambodia, *S. pneumoniae* was the fourth most common pathogen after *Salmonella enterica*, other Gram-negative pathogens and *S. aureus* [7]. Instead, *S. pneumoniae*

was involved in only a minor proportion of BSI in adults in Thailand[3], Laos[2] and Vietnam[4]. This contrasts with sub Saharan Africa[8] where higher (measured) incidences of invasive pneumococcal disease among children and adults have been observed - attributed to high HIV-prevalence and/or less intense antibiotic use in the community.

These local and population-related spectra of etiology of BSI are to be taken into account when developing local treatment guidelines. Systematic reviews with datasets compiled from broad geographical areas over large time periods may lead to dilution of data with loss of the relevant regional differences, patterns and trends. Although regional reviews may have an important role as 'whistleblower', we believe that future priority should be given to interactive country maps, fed by locally generated surveillance data such as *e.g.* EARS-net [9, 10].

Resistance: Pandora's box?

Either due to intrinsic or acquired resistance mechanisms, a large proportion of the pathogens cultured in Cambodian patients with BSI were notoriously 'difficult-to-treat', requiring treatment with expensive broad spectrum or second line antibiotics such as ceftazidime, carbapenems or vancomycin. This is especially worrisome within the context of a poor country such as Cambodia where most health care facilities lack the necessary diagnostic and therapeutic means and where knowledge of antibiotic resistance is limited among health care workers, policy makers and the general public.

Among the largest group of BSI pathogens, *E. coli* and other *Enterobacteriaceae* (Chapter 5), the most prominent finding was that about half of these pathogens carried ESBL of the 'pandemic' CTX-M type, and 70% of those were co-resistant to at least three alternative antibiotics. In addition, we noted a wide variety of concurrent other beta-lactamases. This probably reflects the large pool of resistance genes present in the population and in the environment, which is selected out by high antibiotic pressure and from where a continuous exchange and recombination of resistance genes can occur. In our study population, the prior use of third-generation cephalosporins was indeed the main risk factor of ESBL-positive *E. coli* BSI.

While the dissemination of these resistance rates and the expansion of the diagnostic capacity in Cambodia generated more awareness among Cambodian physicians on the prevalence of antibiotic resistance, it is to be feared that, without an accompanying antibiotic policy, this may lead to an overuse of carbapenem antibiotics. As a matter of fact, during the surveillance period 2007-2010 we did not encounter carbapenemase producing *Enterobacteriaceae* (CPE), although over the past few

months two patients with carbapenem-resistant *Klebsiella pneumoniae* were diagnosed at SHCH [11]. This highlights the fact that the 'new wave' of antibiotic resistance, already intensely present in other countries of South and Southeast Asia [12], has arrived in Cambodia as well.

However, our study comparing outcome of patients with ESBL-positive and ESBL-negative *E. coli* BSI suggested that patient co-morbidity and severity of illness were probably more important determinants of mortality than the resistance pattern or appropriateness of the empirical antibiotic therapy per se. This would imply that it is urgent to invest in better sepsis care and that carbapenem antibiotics should not be used indiscriminately as empirical treatment. But, as is clear from the fierce international debate upon the impact of resistance patterns and empirical antibiotics for *E. coli* BSI [13, 14], more information from larger and prospective studies is needed in Cambodia and other LMIC.

In contrast, for patients with melioidosis (Chapter 3) we did observe an increased mortality in those who had not received appropriate empirical treatment. *Burkholderia pseudomallei* isolates did not display acquired resistance stricto sensu (*i.e.* for the preferred antibiotics ceftazidime, amoxicillin-clavulanic acid or SMX-TMP) but the organism itself has intrinsic resistance to a wide range of commonly used antibiotics (including amoxicillin, ceftriaxone, ciprofloxacin and aminoglycosides) so that in practice it should also be considered as 'difficult-to-treat', particularly since it occurs in the community setting. Increasing the proportion of melioidosis patients to whom appropriate empirical treatment would be given in time would imply (a) a higher index of suspicion, (b) clinical tools to identify those most at risk, (c) supportive diagnostic facilities and (d) the inclusion of an antibiotic with known anti-melioidosis activity in standard treatment guidelines for its most common and severe presentations: community-acquired pneumonia (CAP) and septic shock, as was done *e.g.* in northern Australia [15].
During the past five years of clinical experience with melioidosis in SHCH, we saw a significant decrease in mortality along increasing proportions of patients with adequate antibiotic therapy [16] (Figure 1). Dissemination of results and several workshops have contributed to more awareness on melioidosis among health care workers in the entire country, but the availability of a timely diagnosis and effective treatment nationwide remains a challenge.

Resistance patterns of *Salmonella* spp. (Chapter 4) were different from those in other *Enterobacteriaceae*. *Salmonella* Typhi, the cause of typhoid fever and affecting generally healthy children and young adults, displayed no ESBL but high rates of multidrug resistance and decreased

ciprofloxacin susceptibility. Decreased ciprofloxacin susceptibility was associated with classical point mutations in gyrase A, which is in line with findings from neighboring countries *e.g.* Vietnam [17]. Recently, our findings have been confirmed in other bloodstream infection studies in the general population and in children in Cambodia [18, 19]. The presence of fluoroquinolone resistance has more implications for the management of *Salmonella* Typhi than for other *Enterobacteriaceae*, as it has become the drug of choice for the treatment of typhoid fever since the late 1980's after emerging resistance to chloramphenicol and other first line antibiotics, and because of the drug's capability of intracellular killing and reducing relapses [20, 21]. The actual high rates of decreased ciprofloxacin susceptibility, which may lead to clinical failure in at least 40% of cases [22, 23], would imply a switch to 'next line' antibiotics such as ceftriaxone or azithromycin, both drugs which are associated with resistance problems in other pathogens. Fortunately, the *Salmonella* Paratyphi A strains which are currently circulating and causing an outbreak of paratyphoid fever in Phnom Penh and surrounding areas did not display extensive resistance [24, 25], although in depth study is still pending, and further close surveillance of resistance rates is warranted.

Instead, non-typhoid *Salmonella* spp, zoonotic pathogens affecting mainly severely immune depressed patients displayed more resistance *e.g.* for azithromycin and third generation cephalosporins (due to ESBL) in addition to high rates of resistance for ampicillin, SMX-TMP, chloramphenicol and ciprofloxacin. This may influence particularly the empirical treatment in severely immune depressed patients presenting with fever.

The pig-related pathogens *Salmonella* Choleraesuis (Chapter 4) and *Streptococcus suis* (Chapter 7) were found as causative agents in invasive infections, BSI and meningitis among Cambodian patients. They display remarkable rates of macrolide resistance including for azithromycin. This may be due to the frequent use of tylosin and other macrolides in the veterinary sector [26, 27]. The findings of highly resistant zoonotic pathogens highlights the importance of a 'One Health' concept while assessing the burden and influencing factors of antibiotic resistance in a country. In that regard, these high rates of azithromycin resistance reveal the vulnerability of the presumed 'reserve' antibiotics such as azithromycin and colistin, both of which are liberally used in animal husbandry in Cambodia, Vietnam and probably throughout the region [28-30]. Such findings warrant urgent planning of integrated human-animal surveillance of resistance and antibiotic use (*e.g.* for key pathogens such as *Salmonella* spp. , *Campylobacter* spp., *E. coli* and *S. aureus*), and the opening of the debate, also in LMIC, on antibiotic policies in the animal health sector, where antibiotic use is known to surpass the use in humans [31, 32].

Finally, the in-depth study of *Staphylococcus aureus* causing BSI (Chapter 6) highlighted the wide variety of molecular and resistance types and the increasingly blurred borders between community-acquired and hospital-acquired resistance. We also found links to the new category of livestock-associated MRSA. The MRSA rate of 25% is a concern, but gives -in our opinion- insufficient indication to position a second line antibiotic such as vancomycin in the empirical treatment of complicated SSTI. Instead, similar to our findings in *E. coli* BSI, we found that severity of illness and patient-related factors have more influence on mortality than resistance pattern or appropriate empirical treatment. Therefore we think that priority should be given to improved case management for every *S. aureus* BSI, including proper initial sepsis care. In addition, the use of an adequate antibiotic in a correct dose and duration, the search and removal of infectious foci and close clinical and microbiological patient follow-up should be established on a routine basis.

Strengths and limitations

As noted in the separate chapters, the main limitations of this study were the relatively small numbers of patients and the short follow-up time, which may have caused underestimation of morbidity and mortality. In addition, the large proportion of patients with co-morbidity and the study's 'snapshot' character may impede its representativeness for the general population of Cambodian adults. In particular the retrospective nature of the in-depth data collection has lead to the limited availability of certain clinical variables, which may have jeopardized the strength of certain parameters for predicting the presence of certain pathogens or resistance patterns.

HIV-infection and diabetes mellitus were common in our study population (16% and 9% respectively) and this was reflected in different etiological spectra (*i.e.* we noted relatively more *Salmonella* Choleraesuis and *S. aureus* in patients with HIV-infection and more *B. pseudomallei* in diabetes patients) but not in different resistance patterns for the ubiquitous pathogens [33], nor did we find these co-morbidities to be a significant risk factor for the acquisition of ESBL *E. coli* or MRSA. In addition, recent reports from starting-up laboratories in provincial hospitals across Cambodia confirm the bacterial spectrum and the resistance trends we described in this work.

Another limitation is that in this first phase of bacterial surveillance, we focused only on eubacterial pathogens and did not look for other possible causes of (severe) febrile illnesses: mycobacteria, yeast and fungi, dengue and other viruses and other zoonotic bacteria such as *Rickettsia* spp.and *Leptospira* spp.. Recent studies on causes of fever in Cambodian children [34] and in the general

population [35] suggest that influenza, dengue and Japanese encephalitis viruses and *Orientia* and *Rickettsia* spp. play an important role in the differential diagnosis of febrile illnesses. Similar observations have been made in neigbouring countries [36, 37] although not all diagnostic criteria for these infections are clear-cut. If confirmed, these findings may have an impact on treatment guidelines as they may require particular antibiotics (*e.g.* doxycycline for *Rickettsia*) or no antibiotics at all but particular clinical care in the case of for instance dengue hemorrhagic fever. Validated diagnostic tools are also needed to differentiate between these diagnoses and 'classical' invasive bacterial infections. In a response to the broader need for improved diagnostic means for infections in tropical LMIC, the global research network for better Diagnosis in Neglected Infectious Diseases [38] was created in December 2010, in which both SHCH and ITM are partners and collaborate in research for better diagnostic aids in patients with chronic fever. It is to be hoped that the upcoming results may shed more light on the particular prevalence of each of the aforementioned etiological agents.

Finally, while blood cultures have excellent specificity for the diagnosis of invasive bacterial infections, they have low sensitivity. As the backbone of our study was surveillance of blood cultures and other sterile or non-contaminated samples (*e.g.* CSF, pus from closed abscesses), we may have diagnosed only the tip of the iceberg of certain invasive bacterial infections. For instance, it is well-known that only about 10-30% of patients with a community-acquired pneumonia (CAP) do present with concomitant BSI [39, 40]. Therefore, treatment guidelines covering the entire range of CAP would require also information on the etiology of non-bacteremic CAP. In addition, insight in the etiology of CAP in patient populations at the different care levels (primary care, provincial and referral hospitals) is needed as a base for treatment guidelines. Therefore, in June 2012 the ITM-SHCH collaboration joined colleagues from other health care and research settings in Cambodia in the Community-Acquired Lung Infections, Bacteria & Antibiotics Network (CALIBAN) [41] with the aim to provide Cambodian health policy-makers with evidence-based information on bacterial etiologies of community-acquired pneumonia in Cambodia and their antimicrobial resistance levels, using both published and unpublished data. This compilation of data on the etiology of CAP from all different health care levels in Cambodia and from the neighboring countries suggests that *Streptococcus pneumoniae* and *Haemophilus influenzae* are indeed the most important causes of CAP in children and adults, but difficult-to-treat Gram-negative pathogens such as *B. pseudomallei* , ESBL-positive *Klebsiella pneumoniae* and even non-typhoid *Salmonella* occur frequently in older or immune depressed patients with CAP. The scarcely available data on penicillin susceptibility in *S. pneumoniae*

suggest that about 25% of pathogens displays high-level resistance, and another 30% is intermediately resistant to penicillin. Information on the role of atypical pathogens causing CAP in Cambodia has been limited so far [42].

On the side of study strengths, we think that the association of microbiological, molecular and clinical data had added value in improving the understanding of the local epidemiology of the particular pathogens involved in invasive infections. Their complementary information helped for instance to conclude that *Salmonella* Choleraesuis causes relapsing disease in AIDS-patients, that the observed peak of *S. suis* cases in 2010 was not a focal outbreak but separate infections closely linked to circulating *S. suis* in Vietnam, that PVL-positive MSSA is a very common cause of severe skin and soft tissue infections in Cambodia and that the expansion of ESBL-*E. coli* in Cambodia is heterogeneous and not exclusively caused by the spread of the successful clone CTX-M-15-positive *E. coli* ST 131. Future use of techniques with even higher 'resolution' such as whole genome sequencing may help to understand better the dynamics of bacterial infections and spread of resistance, although the generation and interpretation of these data is time consuming and requires solid bio-informatics systems [43]. On the other hand, when (financial) choices are to be made, it is to be taken into consideration that in a low-income country such as Cambodia, investing in a solid network of quality-assured basic microbiology laboratories across the country may have a better short-term impact on patient care and outcomes than establishing high level laboratories in the capital only.

Finally, our findings were derived from a 'real live' study, most of the data were the first of their kind to be reported in Cambodia. They have triggered awareness and a set of activities at SHCH and at national level. At SHCH, the data were used for teaching and were the start of antibiotic stewardship activities (see below). But however relevant the achievement, we observed also ample room for improvement and continued assessment of the quality of the generated data. For instance, two assessments of the real blood volumes cultured (2012, 2013) revealed that bottle under filling occurred in about 40% of samples; this information was fed back to the nurses on several educative occasions and will be closely followed up. Likewise, we observed important fluctuations of the contamination rates. As illustrated in Figure 2, in the initial phase (2007-2010) average contamination was as high as 8.7%, in spite of regular feedback of those data to local health care workers. Only from 2012 onwards, we observed a drop in contamination rates after closer involvement of nurses, intense feedback sessions and the involvement of SHCH staff in teaching

sessions for provincial hospitals. Contamination is now locally perceived as a quality parameter and considered as a tool for improvement. The change between BHI and BacTAlert system did not appear to have a major influence on the contamination rates nor on the spectrum of contaminants. The 'time to reporting' was subject to several changes in the local organization and data flow. In the initial surveillance phase (2007-2010), file revision and ward rounds revealed that reporting of results could take up to 7 days, especially in case of 'difficult' identifications or antibiograms (such as *B. pseudomallei*, or ESBL+ *E. coli*). This was improved by (1) the introduction in 2009 of daily laboratory visits by a dedicated clinician keeping a logbook of all positive blood cultures including the treatment and follow-up of patients, (2) clear guidelines for the laboratory technicians on reporting preliminary and final results and (3) ward access to laboratory results through the hospital information network (Table 1). An assessment of these interventions' impact is ongoing.

8.2. Implications for patient care and public health in Cambodia

Under the impulse of SHCH and ITM , a First National Workshop on the Containment of Antibiotic Resistance was organized in November 2011 in the capital Phnom Penh, in collaboration with the Ministry of Health, WHO and other local partners (Figure 3). It gathered about 200 national and international participants including experts, policy makers and key staff from different hospitals within the country to discuss the issues about antibiotic resistance patterns recently identified in the country [44]. Research findings were published in international-peer reviewed journals but also -in popularized version- in the Cambodian Journal for Nurses and Midwives (Figure 4) in order to reach a large population of health care workers.

As generating awareness among policy makers and health care professionals in Cambodia is an essential first step, more nation-wide interventions are needed to contain antibiotic resistance and to improve patient outcomes in the country, as summarized in Figure 5.

Further expansion of the microbiology laboratory capacity and surveillance

The present as well as others' recent findings on high levels of antibiotic resistance in Cambodia have made clear the need for quality-assured microbiological diagnosis and surveillance of etiological trends and resistance patterns, both for the sake of the individual patient and for the community at large. However, this requires investment in infrastructure, consumables and trained staff. In 2011, the ITM-SHCH team joined a pilot collaboration between NGO's, the Ministry of Health, WHO Cambodia and the University of Health Sciences supporting the roll-out of blood

culture-based diagnosis in five provincial hospitals. The project was launched in February 2013 with a dedicated hands-on workshop for local clinicians, nurses and laboratory technicians (Figure 6); locally produced blood culture vials and close follow-up will hopefully contribute to long-term sustainability.

As recommended by the WHO, future surveillance activities should not only contain data on bacterial resistance, but also on antibiotic use, and should ideally be linked to species-related morbidity and mortality. Quantitative data on antibiotic use in Cambodia are largely lacking. Together with local and international partners, we initiated in August 2013 a nation-wide study on factors influencing antibiotic use in Cambodia (including a Knowledge Attitudes and Practices-survey, Focus Group Discussions and in-depth interviews) which will hopefully help to identify the major drivers influencing the prescription, dispensing and use of antibiotics in men and in livestock in Cambodia and design major stewardship interventions.

Antibiotic stewardship

The set of activities and policies to improve the rational use of antibiotics is known as antibiotic stewardship (or antibiotic policy). Its combined goals are: improving patient outcomes, containment of antibiotic resistance and increased cost-effectiveness of patient care. Essential elements of an antibiotic policy include a stable and restrictive list of antibiotics in use, standard treatment guidelines, audit and feedback of prescriptions, surveillance of bacterial resistance and antibiotic use and education at all levels [45]. Antibiotic stewardship activities have typically been developed in the hospital context, but they should be expanded and adapted to primary care settings as well.

The antibiotic stewardship initiative in SHCH focused on the creation of a hospital Essential Drugs' List, the redaction of locally adapted Standard Treatment Guidelines (see below), educational sessions and restricted access to broad spectrum antibiotics. In a next phase we have started surveillance of hospital antibiotic use and regular point prevalence surveys to assess prescription patterns and adherence to the Standard Treatment Guidelines. These stewardship activities could also be implemented in other hospitals with microbiological facilities, provided a team of dedicated health care professionals can be created. This will require explicit support from policy makers and hospital managers. More creativity will be needed to adapt stewardship activities for the numerous healthcare settings in Cambodia and other LMIC without a microbiology laboratory.

Antibiotic stewardship at community-level in Cambodia is also essential but remains an even larger

challenge because of the dispersed landscape of antibiotic dispensing and use [46]. Well-designed and locally adapted multifaceted, multilevel intervention programs such as currently ongoing in neighboring Thailand may be the way forward [47]. As a start, and following our study findings, we would suggest to policy makers to demotivate the liberal use of fluoroquinolones, azithromycin and cephalosporins in the community and to ensure and monitor the availability and quality of broad spectrum antibiotics in referral hospitals.

Standard treatment guidelines (clinical practice guidelines)

Standard treatment guidelines have been defined as 'systematically developed statements to assist decisions about appropriate health care for specific circumstances' [48]. When made and used well, they may act as decision aids and contribute to quality and efficiency of medical care. However, guidelines cannot not replace clinical judgment, and injudiciously made or oversimplified guidelines can even be harmful [48]. Therefore, the ideal guideline making process requires as much as possible locally relevant evidence (which is graded upon its evidence base and applicability), a trained multidisciplinary group and an implementation plan [49]. Successful implementation of a new guideline implies also local applicability and acceptability, including a workable format [50]. The sad reality is that in many LMIC treatment guidelines are outdated, unworkable 'dusty booklets stored in warehouses' [51]. On the other hand, good examples of actualized guidelines, based on best available evidence following a sound methodology do exist also in LMIC [52, 53].

In the guideline-writing process at SHCH of the past 6 years, the locally generated evidence -however limited- was used and compared to international standards. Within a multidisciplinary team, a consensus was sought, taking into account local availability of drugs, diagnostics and overall feasibility and acceptability. Data from the local microbiological laboratory and clinical outcome findings were integrated in recommendations per disease and per species. As shown in Table 1, these included for instance the introduction of amoxicillin-clavulanic acid for CAP in diabetes patients, ceftazidime for proven melioidosis, the addition of amikacin to ceftriaxone for empirical sepsis treatment, meropenem for proven ESBL-positive BSI , vancomycin for proven MRSA BSI and azithromycin or ceftriaxone for moderate or severe typhoid fever respectively. The first version has been completed in September 2011 and included guidance on major infectious syndromes such as sepsis, pneumonia, urinary tract infection, meningitis and skin and soft tissue infections as well as a section on surgical antibiotic prophylaxis. A strong participatory approach helped to enhance local ownership, although background knowledge of many participants was limited and finding locally

relevant evidence was difficult. However challenging, establishing and implementing guidelines was feasible within the context of SHCH, a NGO hospital with well-trained staff, diagnostic means and the availability of broad spectrum antibiotics through a drugs donation program.

At national level other problems challenge the guideline-making process: in most referral hospitals diagnostic (microbiological) means are scarce and there is limited availability of broad spectrum antibiotics, unaffordable for the majority of patients with limited health care insurance. In addition, the available evidence is patchy and technical expertise on antibiotic resistance at policy level is limited. Seemingly small changes (*e.g.* the replacement of amoxicillin by amoxicillin-clavulanic acid for CAP in risk groups) may have unforeseen financial and logistic impact and require careful modeling and balancing between over - and under-treatment of certain conditions [54, 55]. There is need for easy, cheap and point-of-care based decision tools to identify those most at need of broad spectrum antibiotics. Last but not least, there may be need for good priority setting, as the Cambodian Ministry of Health has planned a fast revision of more than 161 guidelines since 2011.

Infection control

Whereas antibiotic stewardship is essential to decrease antibiotic pressure and the further selection of resistant strains, infection control measures are needed to prevent transmission of pathogenic bacteria between patients, health care workers and the larger community.

Nosocomial infections do occur also in LMIC settings, probably even at high rates [56, 57], as was illustrated by our experiences with healthcare-associated or nosocomial ESBL-positive or MRSA infections, and by an outbreak of *Burkholderia cepacia* from contaminated intravenous fluids in 2011 [58]. Also in other Cambodian hospitals evidence on the problem of nosocomial infections is emerging [59, 60]. A baseline assessment of infection control measures followed by the publication of hospital infection control guidelines was carried out under impulse of the Ministry of Health in 2010-2011, as a start to meet the needs in the health care system's basic infrastructure and hygiene [44, 61].

Hand hygiene is the most essential element of infection control in health care settings [62]. This can also be effectively implemented in low-resources settings, as was illustrated recently in a multi-centric study carried out by WHO [63]. However, beyond the logistics of the availability of water, antibacterial soap or (preferably) alcohol hand gel, implementing effective infection control in Cambodian hospitals will require awareness and leadership for an activity which does not get enough appreciation from many educated health care workers [64].

Effective prevention of bacterial infections in the community further implies the improvement of basic hygienic measures such as safe drinking water, food safety, and proper sanitation, in addition

to targeted vaccination for bacterial pathogens such as *H. influenzae* (Hib), *S. pneumoniae* and *Salmonella* Typhi [65-67]. Of these, the current immunization program in Cambodia only includes Hib besides diphtheria, pertussis, tetanus, polio, measles and hepatitis B [68]. As for other LMIC, a difficult choice is to be made between expanding the population coverage rate versus broadening the number of vaccines per child.

Improved sanitation is, together with safe water provision, included in the 7th Millennium Development Goal [69] but has remained a 'difficult' topic for policy makers and scientists alike because of its cost and cultural sensitivities [70, 71]. In Cambodia, according to the WHO Western Pacific Regional Office, only 28% of the population had access to an improved sanitation in 2008 [72]. In the era of multidrug resistance, more questions arise as to how wastewater should be processed in order to contain the pool of resistance genes as much as possible.

Education

Even though education alone may not be powerful enough as an intervention, it generates a knowledge environment essential for health care workers to understand and support the antibiotic stewardship programs, and it should be initiated very early in the medical curriculum [73]. The role of up-to-date undergraduate and postgraduate education is even more important in settings with limited access to medical literature. This requires however a 'critical mass' of academic staff with technical expertise in bacterial diseases, antibiotic resistance and antibiotic stewardship, which has not yet been attained after several decades of civil war and political instability. The lack of background knowledge among medical doctors in the country on an infection so relevant in the region such as melioidosis, illustrates this. This academic capacity needs to be (re)built as well, along the expansion and valorization of the microbiology laboratory network in Cambodia. An important initiative was the (re)start of a dedicated postgraduate training program at the University of Health Sciences in Phnom Penh, for medical doctors and pharmacists on laboratory medicine.

With regards to the general public, educational and awareness campaigns may help to generate an 'understanding' environment which can support the prescriber to withhold antibiotics [74]. While hard outcome endpoints remain unclear and difficult to measure, there is a consensus that these campaigns contribute to more careful use of antibiotics.

Antibiotic stewardship in the veterinary sector

Following the observation of high resistance rates in several pathogens with zoonotic link (*i.e.*

Salmonella Choleraesuis, *Streptococcus suis*, MRSA ST 398 and ST9), we think that an in-depth assessment of veterinary antibiotic practices is highly needed in Cambodia. 'Snapshot' assessments of local experts [28, 29] confirm that a wide range of common antibiotic classes is being used liberally for preventive (growth promotion) and curative purposes alike in Cambodian husbandry, including tetracyclines, macrolides, cephalosporins and penicillins, fluoroquinolones and colistin, as is also the case in other Southeast Asian countries [75, 76].

Although well-documented examples exist of a successful ban on prophylactic antibiotic use in animals (*e.g.* in Denmark [77]), this idea is very new for most LMIC, where economies including the agricultural industry are in fast expansion. Also in Cambodia, the pig breeding industry is in evolution from traditional backyard farming to semi-industrialized farming [78], of which the impact on antibiotic use and spread of antibiotic resistance is unknown. Larger scale breeding may enable standardization of assessments and medical interventions, but large stables may also imply a higher risk for large-scale transmission of pathogens between animals [79].

In the above mentioned nationwide study on antibiotic use in Cambodia, a set of qualitative studies will explore knowledge and opinions on antibiotic resistance among key stakeholders of animal health in the country.

Innovation and research

Along the above mentioned policy changes and interventions, more local evidence is needed to understand better the risk factors for acquisition, spread and outcome of these (resistant) invasive bacterial infections in Cambodia as well as the impact of potential interventions on the already tight health care budget. Examples of follow-up research to our presented data would include: (1) a nationwide assessment of the burden of melioidosis morbidity and mortality and its prevalence in specific risk groups *e.g.* diabetes patients, (2) the nation-wide prevalence assessment of *Streptococcus suis* among patients with bacterial meningitis, (3) a prospective, multicentric study on prevalence and factors influencing outcome in ESBL *E. coli* and MRSA infections, (4) piloting the feasibility and impact on outcome of sepsis care bundles in referral hospitals.

Awareness and support at policy level

The above mentioned long list of starting up or suggested interventions require visionary coordination at policy level. Comparable to other LMIC, Cambodia's health system is challenged by many other diseases and factors, from maternal mortality over multidrug resistant malaria to traffic accidents, which merit also full priority [44, 80]. In addition, antibiotic resistance is a relatively new, unknown and broad topic for most policy makers, which does not fit into a single 'vertical program';

a situation comparable to the early years of the HIV-AIDS epidemic in the 1990's which was also a complete challenge for the health care system in the affected countries [81].

In November 2012, Cambodia's Ministry of Health created a new 'Antimicrobial Resistance Working group', with representatives of the different national programs in malaria, HIV, tuberculosis, large hospitals, universities, NGO's, the pharmaceutical and veterinary sector and other relevant stakeholders with the aim to jointly assess and discuss the situation and establish a strategic plan. SHCH has been invited to be member of this Working Group. One of the roles of a health care provider such as SHCH could be to be the 'messenger' of the clinical reality, of the needs and constraints in the field including the provision of regular reports on hospital-based surveillance data.

Regional and international collaboration

Finally, it is clear that antibiotic resistance does not stop at national borders, especially not in this era of intense international travel of people, animals and goods. This was well illustrated by our recent report on a presumed outbreak of *Salmonella* Paratyphi A in Cambodia (Chapter 4) which coincided with several observations across Europe [25, 82] of travelers returning from Cambodia with paratyphoid fever. Regional collaboration in surveillance and exchange of experiences would be a first step, but genuine international action is needed to steer and coordinate interventions, somehow comparable to the approach of UNAIDS in the containment of the HIV-AIDS epidemic, or to the Millennium Development Goals. Essential activities would include a worldwide assessment of the most urgent problems, (ambitious) national plans with accountability and mile stones to be reached, and close collaboration of scientific, professional and political communities.

Figure 1. Evolution of mortality and correct empirical/directed antibiotic treatment for melioidosis patients in Sihanouk Hospital Centre of HOPE, 2007-2011. (From: Thong Phe, Erika Vlieghe et al., Five-year experience of diagnosis and treatment of melioidosis in Cambodian adult patients. Poster presented at 7th World Melioidosis Congress, Bangkok, September 18-20 2013)

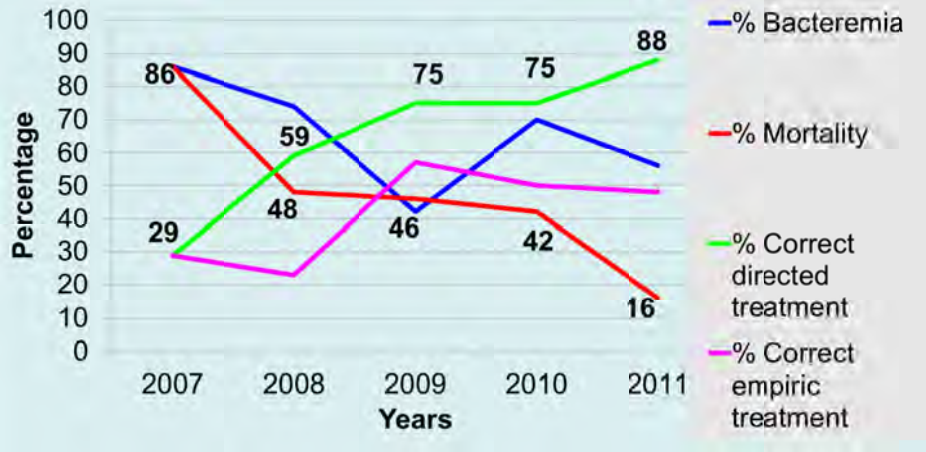

Figure 2. Yield of pathogens and contamination rates in blood culture, SHCH (2007-2013)

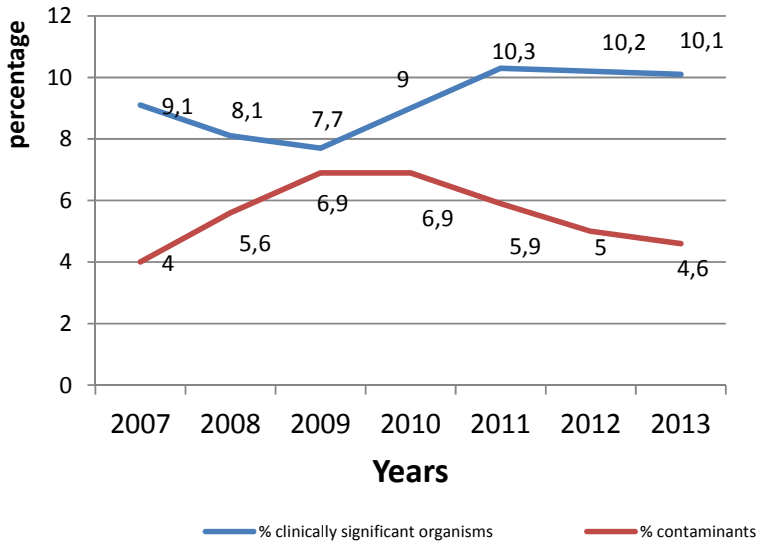

Figure 3. First National Workshop on Antibiotic Resistance (Phnom Penh, November 2011)

Figure 4. Special issue of the Cambodian Journal for Nurses and Midwives on 'Antibiotic resistance' (issue April-June 2012)

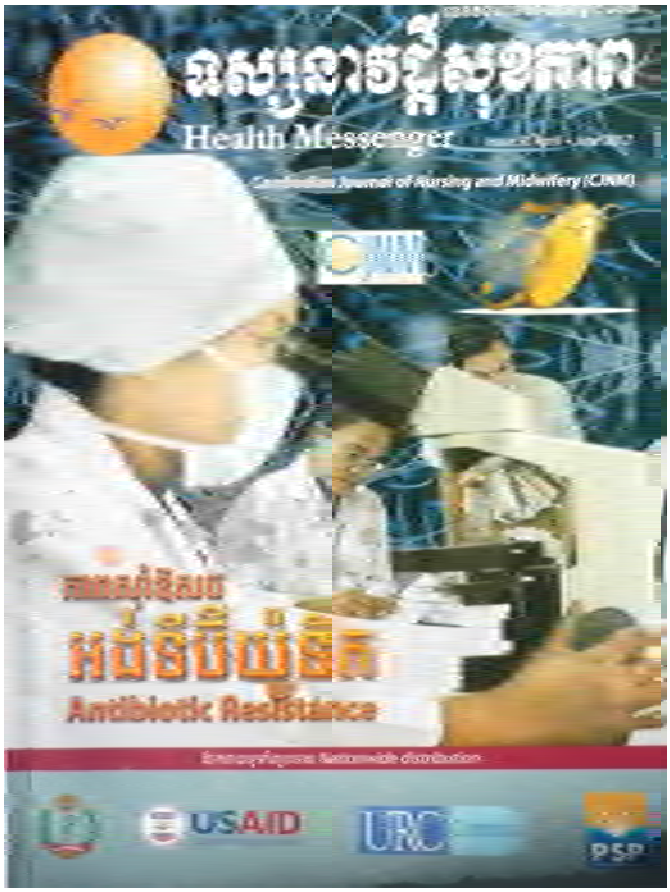

Figure 5. Interventions at patient, dispenser, prescriber and environmental level to contain antibiotic resistance.

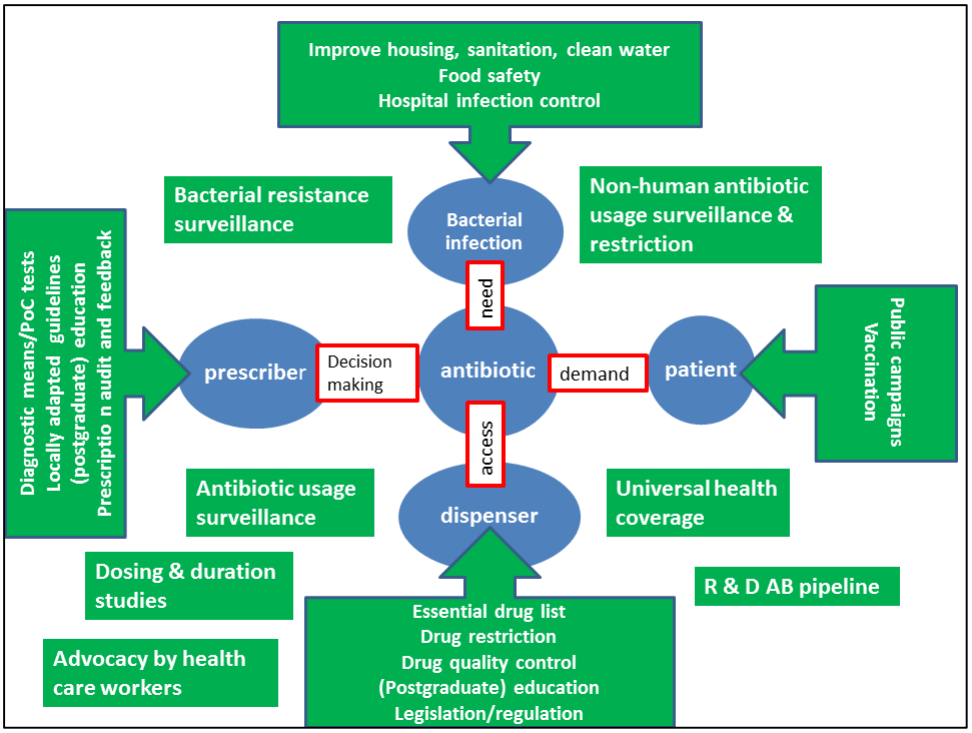

Figure 6. SHCH-clinician Thong Phe trains fellow-clinicians from provincial hospitals in the essentials of blood culture-based diagnosis (Blood culture workshop, Phnom Penh February 2013)

Table 1. Evolution of antibiotic treatment guidelines and hospital antibiotic policy in SHCH 2007-2013

	Before 2007	2007-2010	2010-present	Future challenges
(Blood) culture-based diagnosis	blood cultures taken sporadically ('severely ill bias')	blood cultures in all patients with SIRS	blood cultures in all patients with SIRS	financial sustainability, quality control in multiple clincis
Culture result communication	paper based, delay > 7 d	dedicated 'liaison'-clinicians	dedicated 'liaison'-clinicians, preliminary results by phone, computer/paper based final results	critical number of dedicated clinicians, further integration of LIS and hospital intranet
Treatment guidelines	no uniform guidelines, decisions based on: expert opinion, imported guidelines from expatriate staff, (outdated) national guidelines	SHCH guidelines made and implemented: surgical prophylaxis (3/2009), sepsis (4/2009), CAP (2/2010), UTI (2/2010), endocarditis (5/2010), meningits (5/2010), bone/joint infections (11/2010), SSTI (3/2011)	revision and bundling of all guidelines (8/2011), further adaptions to melioidosis and salmonellosis guidelines (2012)	ensured availability of drugs, evaluation of hospital-wide implementation, regular revision needed, streamline with national guidelines,…
	Common habits	**Key messages from study**	**Key changes for guidelines**	**Future challenges**
sepsis	cephalosporin with or without fluoroquinolone or gentamicin	information on antibiotic per presumed focus + sepsis care needed	AB advice specified per focus, early goal directed sepsis care included	quality of sepsis care to be assessed, alternatives for penicillin allergy are scarce
pneumonia	low dose amoxicillin or amoxicillin-clavulanic acid, cefuroxim, fluoroquinolones, ceftriaxone	increase amoxicillin dosing, include empirical coverage for melioidosis in risk groups, ban fluoroquinolones	CAP 1: high dose (oral) amoxicillin, CAP 2-3: amoxicillin-clavulanic acid, CAP 4: include ceftazidime if proven melioidosis	availability of ceftazidime, IV amoxicillin-clavulanic acid, alternatives for allergic patients,…
intra-abdominal infection	ciprofloxacin, ceftriaxone or ceftazidime, metronidazole, gentamicin	cover empirically for ESBL+ *Enterobacteriaceae* in severely ill	empirical amoxicillin-clavulanic acid (or piperacillin-tazobactam) +/- amikacin*; meropenem for proven ESBL+ infections	(un) availability of amoxicillin-clavulanic acid, piperacillin-tazobactam, amikacin and colistin
UTI	ciprofloxacin, SMX-TMP, ceftriaxone	cover empirically for ESBL+ *Enterobacteriaceae* in severely ill, include alternative drugs for mild cases	mild: nitrofurantoin; urosepsis: amoxicillin-clavulanic acid (or piperacillin-tazobactam) +/- amikacin*; meropenem for proven ESBL+ infections	managment of recurrent UTI, alternative drug for nitrofurantoin (e.g. fosfomycin), availability of amoxicillin-clavulanic acid, amikacin
typhoid fever	ceftriaxone, ciprofloxacin, amoxicillin-clavulanic acid	ban use of ampicillin or SMX-TMP, avoid empiric use of ciprofloxacin	mild: ceftriaxone empirically, adapt to ciprofloxacin or azithromycin	
meningitis	ceftriaxone normal dose, amoxicillin-clavulanic acid	*Streptococcus suis* is an important pathogen	high dosed ceftriaxone + dexamethasone, consider need for prolonged treatment	
SSTI	lincomycin, vancomycin, low dose oral cloxacillin	include high dosed cloxacillin, restrict vancomycin for MRSA only, avoid lincomycin, include coverage for melioidosis in severe cases	flucloxacillin IV and PO is backbone, vancomyin restricted for MRSA, amoxicillin-clavulanic acid if suspicion of melioidosis	ensured availability of IV cloxacillin, ? SMX-TMP as alternative for vancomycin
Essential Drug List (EDL)	random donations (ceftazidime, ertapenem, vancomycin, PO amoxicillin-clavulanic acid); wide variety of PO formulations; absence or limited availability of non-donated first line drugs e.g. IV cloxacillin, limited guidance on use of different antibiotics	uniformization of formulations, ensured availability of IV cloxacillin, vancomycin, ceftazidime, amoxicillin-clavulanic acid, amikacin	'guided' donation or purchase of meropenem, cefepime, vancomycin, amoxicillin-clavulanic acid for restricted use only	sustained availability of second/third line antibiotics, streamlining with national drug policies, critical number of dedicated pharmacists,...

** not yet fully implemented, awaiting final approval of hospital's antibiotic committee; CAP: community-acquired pneumonia; UTI: urinary tract infection; SSTI: skin and soft tissue infection*

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Summary

Invasive bacterial infections, including bloodstream infections, are a major cause of morbidity and mortality around the world, as explained in **Chapter 1**. In order to choose the most adequate empiric antibiotic, clinicians require information on the most frequent bacteria causing invasive infections. This information can be obtained by culturing blood from patients suspect of invasive bacterial infections (*i.e.* blood cultures). Given the worldwide spread of antibiotic resistance, invasive bacterial infections have become more difficult-to-treat with commonly available antibiotics. Therefore it is also important to measure resistance rates among invasive bacteria. In addition, studying the (genetic) mechanisms behind antibiotic resistance and the genetic relations between bacteria can contribute to a better understanding of how bacteria become resistant and spread among people and in the environment. In high-income settings with a sufficient number of high-quality microbiological laboratories such as Europe and Northern America, information on the presence of (resistant) bacteria is widely available through surveillance systems, based on nationwide networks of quality-assured laboratories. In low- and middle income countries such as Cambodia, well-functioning laboratories are very scarce, and essential information on the causes and resistance patterns of invasive bacterial infections is often not available.

In 2007, the Institute of Tropical Medicine, Antwerp (ITM) and Sihanouk Hospital Centre of HOPE (SHCH), Phnom Penh, Cambodia, started a joint study of blood cultures taken from all adult patients who presented with fever in the hospital between 2007 and 2010. During this study period, 5714 blood culture samples were taken from 4833 patients; in 8.8 % (n = 445) of these samples we detected pathogenic bacteria (**Chapter 2**). Nearly one out of four patients with a bloodstream infection died. The most common and remarkable bacteria were *Escherichia coli* and other *Enterobacteriaceae*, *Salmonella enterica, Burkholderia pseudomallei, Staphylococcus aureus* and *Streptococcus suis*; for all of which we found high resistance rates to several commonly used antibiotics. A detailed description of the diseases caused by these bacteria and their resistance patterns is given in Chapters 3 to 7.

Burkholderia pseudomallei **(Chapter 3)** is a bacterium living in soil and water, mainly in Southeast Asia and northern Australia. It is the cause of the disease melioidosis, which presents in many different ways such as a skin, lung, bone or bloodstream infection. The bacterium has intrinsic resistance against many commonly used antibiotics and requires treatment with expensive, broad spectrum antibiotics. We described 58 patients with melioidosis in Cambodia. The disease was

mainly seen in patients with diabetes mellitus and during the rainy season. More than half of the patients died, especially those with a severe condition such as a bloodstream infection (RR 6.8 (1.82-25.5) and those who received inappropriate antibiotic treatment (RR 3.5 (2.07-5.90), $p < 0.001$). An earlier diagnosis and the availability of effective antibiotics would be a step forward in better outcomes for these patients.

Salmonella bloodstream infections **(Chapter 4)** can be caused by *Salmonella* types which infect only humans (*Salmonella* Typhi and *Salmonella* Paratyphi which cause enteric fever), or by non-typhoid *Salmonella* types which cause illness in animals and only occasionally in humans. Between 2007-2010 we described 72 patients with *Salmonella* bloodstream infections. These were caused by *Salmonella* types of the first group – *Salmonella* Typhi (20 cases) and *Salmonella* Paratyphi (2 cases) – and by 'non-typhoid' *Salmonella* types, most commonly *Salmonella* Choleraesuis (50 cases). This bacterium causes fever in pigs and occasionally in humans with very low immunity. In patients with HIV/AIDS, *Salmonella* Choleraesuis caused multiple episodes of fever. All *Salmonella* presented with high resistance rates, especially for ciprofloxacin (in *Salmonella* Typhi) and azithromycin (in *Salmonella* Choleraesuis), two important antibiotics for the treatment of enteric fever. Therefore, a review of the guidelines for enteric fever treatment is warranted.

Surprisingly, between 2011 and 2013, we observed a sudden and very sharp rise in the number of infections with *Salmonella* Paratyphi A, mostly in the capital Phnom Penh. As this coincides with an increased number of European travelers returning from Cambodia with enteric fever, we presume a local outbreak is ongoing which warrants prompt investigation and control measures.

Escherichia coli (**Chapter 5**) is the most common cause of (complicated) intra-abdominal and genito-urinary infections. In our study, about 50% of these bacteria were highly resistant for a combination of first and second line antibiotics, mostly due to the presence of extended spectrum beta-lactamases (ESBL). Most of these highly resistant bacteria with ESBL carried a common mechanism (*i.e.* CTX-M) which is spreading quickly around the world, including in Cambodia. In our study, people who had recent exposure to antibiotics were at higher risk of having a ESBL-positive *Escherichia coli* bloodstream infection (RR 1.46 (1.03-2.09), $p = 0.035$). About 30% of all patients with *Escherichia coli* bloodstream infection died, especially those who suffered from many other illnesses (RR 2.75 (1.11-6.81), $p = 0.028$) such as chronic liver disease. Remarkably, inappropriate antibiotic choices did not increase mortality significantly (RR 1.16 (0.66-2.06), $p = 0.669$), in contrast with melioidosis patients.

Staphylocccus aureus **(Chapter 6)** is the most common cause of skin infections worldwide. Its most famous resistance type is methicillin resistance ('MRSA'), known as a typical nosocomial pathogen, but now frequently associated with community-acquired infections as well. In our study, 23% of invasive *Staphylococcus aureus* infections was of the MRSA-type. Older age, superficial skin

infections and recent hospital contact were risk factors for infection with MRSA. About 15% of all patients died, especially those older than 50 years of age (RR 4.27 (1.14-15.9), p = 0.044). We observed a wide variety of genetic *Staphylococcus aureus*-types, but five main clones dominated, including 2 types which have been found in animals as well (*i.e.* ST 398 and ST 9).

During the study period, we observed also 13 patients with invasive *Streptococcus suis* infection **(Chapter 7**). *Streptococcus suis* usually infects pigs; people can acquire the pathogen through close contact with pigs or undercooked food. These 13 patients presented with meningitis with or without bloodstream infection and often required antibiotic treatment during several weeks. All patients survived, but one third had complications such as deafness. Interestingly, we noted important similarities with *Streptococcus suis* isolates circulating in southern Vietnam and found resistance problems associated with antibiotic use in animals.

In **Chapter 8** we concluded that bloodstream infections in Cambodian adults are often associated with high mortality and high levels of complex antibiotic resistance. Therefore, several urgent measures are to be taken. To contain antibiotic resistance we suggest a wide range of actions, from an improved availability of necessary drugs, adapted treatment guidelines and hand hygiene to updated education and international collaboration.

Samenvatting

Invasieve bacteriële infecties, zoals infecties waarbij bacteriën in de bloedbaan komen ('bloedbaaninfecties'), veroorzaken wereldwijd veel ziekteleed en sterfte (**Hoofdstuk 1**). Achtergrondkennis over de meest voorkomende bacteriën die deze invasieve infecties veroorzaken, is voor artsen essentieel om het meest geschikte antibioticum voor hun patient te kunnen selecteren. Bacteriële infecties zijn immers veel moeilijker behandelbaar geworden door de wereldwijde opmars van antibioticaresistentie. Daarom is het ook belangrijk om de aanwezigheid van antibioticaresistentie te meten in bacteriën die invasieve ziekte veroorzaken. Daarbij is het ook nuttig de (erfelijke) mechanismen die bacteriën resistent maken en hun onderlinge verhoudingen te bestuderen. Bacteriële kweek van het bloed van erg zieke patiënten ('bloedkweken') kan heel wat interessante informatie over ziekteverwekkers en hun resistentiepatronen aanleveren. In rijke landen met voldoende microbiologische laboratoria van goede kwaliteit, zoals in Europa en Noord-Amerika, is deze informatie beschikbaar via zogenaamde 'surveillance' systemen, netwerken van kwaliteitslaboratoria. In armere landen, zoals Cambodia, zijn goed werkende laboratoria erg schaars, waardoor heel weinig bekend is over lokale oorzaken van invasieve bacteriële infecties en hun resistentiepatronen.

In 2007 startten het Instituut voor Tropische Geneeskunde (Antwerpen) and Sihanouk Hospital Centre of HOPE (SHCH) in Phnom Penh, Cambodia, een gezamenlijke studie van bloedkweken bij volwassen patiënten die zich met koorts in SHCH aanmeldden. In de periode 2007-2010 werden 5714 bloedstalen voor kweek afgenomen bij 4833 patiënten. In 445 stalen (8.8%) detecteerden we ziekmakende bacteriën (**Hoofdstuk 2**). Ongeveer één op vier patiënten met bloedbaaninfectie overleed. De meest voorkomende en opmerkelijke bacteriën waren: *Escherichia coli* and andere darmbacteriën, *Salmonella enterica, Burkholderia pseudomallei, Staphylococcus aureus* and *Streptococcus suis*. Veel van deze bacteriën waren resistent voor verschillende vaak gebruikte antibiotica. In Hoofdstukken 3 tot 7 geven we een meer gedetailleerde beschrijving van de ziekten die deze bacteriën veroorzaken en van hun resistentiepatronen.

Burkholderia pseudomallei **(Hoofdstuk 3)** is een bacterie die leeft in de bodem en in het oppervlaktewater in specifieke streken op aarde, vooral in Zuidoost-Azië en in het tropische Noorden van Australië. Deze bacterie is de verwekker van 'melioïdose', een infectieziekte die aantasting en abcesvorming ter hoogte van de huid, longen, lever, milt en ook bloedbaan kan veroorzaken. De bacterie heeft een 'aangeboren' resistentie voor de meest gangbare antibiotica;

doeltreffende behandeling dient te gebeuren met (vaak dure) breedspectrumantibiotica. In onze studie beschreven we een reeks van 58 patiënten met melioïdose in Cambodia. De ziekte manifesteerde zich vooral tijdens het regenseizoen, en voornamelijk bij mensen met onderliggende suikerziekte. Meer dan helft van de patiënten overleed, vooral patiënten met ernstige vormen van de ziekte (bv. bloedbaaninfectie) en zij die initieel een foute antibioticatherapie kregen. Een snellere diagnostiek en de beschikbaarheid van doeltreffende antibiotica zouden een stap voorwaarts betekenen voor de behandeling van deze patiënten.

Salmonella bloedbaan infecties **(Hoofdstuk 4)** kunnen veroorzaakt worden door *Salmonella* types die alleen mensen infecteren (met name *Salmonella* Typhi en *Salmonella* Paratyphi, die beiden buiktyfus veroorzaken), of door 'niet-tyfoïde *Salmonella'*, ziekteverwekkers van dieren die af en toe ook mensen ziek maken. Tussen 2007 en 2010 beschreven we 72 patiënten met *Salmonella* bloedbaan infectie: 20 en 2 werden respectievelijk veroorzaakt door *Salmonella* Typhi/Paratyphi, en 50 door 'niet-tyfoïde *Salmonella',* voornamelijk *Salmonella* Choleraesuis. Deze bacterie veroorzaakt gewoonlijk koorts bij varkens, en af en toe bij mensen met een zeer lage immuniteit. In onze studie veroorzaakte *Salmonella* Choleraesuis herhaalde koortsepisodes bij mensen met HIV/AIDS. Antibioticaresistentie was hoog bij de meeste van deze *Salmonella*-infecties, vooral dan voor ciprofloxacine (bij *Salmonella* Typhi) en voor azithromycine (bij *Salmonella* Choleraesuis), twee belangrijke antibiotica in de behandeling van buiktyfus. Daarom stellen we voor de lokale behandelingsrichtlijnen voor deze infecties aan te passen.
Tot onze verrassing zagen we tussen 2011 and 2013 een zeer sterke stijging van het aantal infecties door *Salmonella* Paratyphi A, vooral in de hoofdstad Phnom Penh. Aangezien dit samenviel met de observatie in een aantal Europese landen dat er ook een forse toename was van uit Cambodia terugkerende reizigers met *Salmonella* Paratyphi A-infectie, vermoeden we dat zich een lokale epidemie voordoet, waarvoor verder onderzoek vereist is.

Escherichia coli (**Hoofdstuk 5**) is de meest voorkomende verwekker van urineweg- en buikinfecties. In onze studie stelden we vast dat 50% van deze bacteriën erg resistent zijn, voornamelijk door aanwezigheid van een specifiek resistentie type genaamd 'extended spectrum beta-lactamase' (ESBL). Bijna al deze hoogresistente bacteriën met ESBL bezaten hetzelfde mechanisme, genaamd 'CTX-M', waarvan gekend is dat het zich de laatste jaren erg succesvol wereldwijd verspreid heeft. Mensen die reeds thuis antibiotica genomen hadden voor ze naar het hospitaal kwamen, hadden in onze studie een hoger risico om ESBL-positieve *Escherichia coli* bloedbaaninfectie te krijgen.

Ongeveer één op drie patiënten met *Escherichia coli* bloedbaaninfectie stierven, vooral mensen met andere onderliggende ziekten (zoals chronisch leverlijden). Opmerkelijk genoeg had een foute antibioticakeuze hier niet dezelfde slechte invloed op overleving als bij melioïdose patiënten. *Staphylocccus aureus* **(Hoofdstuk 6)** is de belangrijkste verwekker van huidinfecties wereldwijd. De meest voorkomende resistentie in deze bacterie is resistentie voor methicilline ('MRSA'). MRSA is algemeen gekend als een typische 'hospitaalbacterie', maar komt nu ook meer en meer buiten hospitaalcontext voor. In onze studie was 23% van alle invasieve *Staphylococcus aureus* infecties MRSA. Hoge leeftijd, oppervlakkige huidinfecties en een recent contact met de gezondheidssector bleken risicofactoren voor infectie met MRSA. Ongeveer 15% van alle patiënten overleed, vooral mensen ouder dan 50 jaar. We stelden tenslotte een brede waaier een genetische types van deze bacterie vast, maar merkten toch vijf 'dominante' types op, waarvan er twee ook gekend zijn als ziekteverwekker bij dieren.

Tijdens onze studieperiode stelden we ook de diagnose van invasieve *Streptococcus suis*-infectie bij 13 patiënten **(Hoofdstuk 7**). *Streptococcus suis* infecteert normaal gesproken varkens; mensen kunnen per toeval besmet raken door nauw contact met varkens or onvoldoende bereid vlees. Deze ziekte komt veel voor in Vietnam en het Verre Oosten. Deze 13 patiënten hadden hersenvliesontsteking met of zonder bloedbaaninfectie, en hadden vaak verschillende weken antibiotica therapie nodig om te genezen. Al deze patiënten overleefden hun infectie, maar één op drie hield er restletsels zoals doofheid aan over. Het resistentiepatroon van deze 'varkensbacteriën' deed vermoeden dat er veel antibiotica aan deze dieren gegeven wordt. Tenslotte stelden we ook vast dat de *Streptococcus suis*-bacteriën uit Cambodia genetisch erg lijken op deze uit Zuidelijk Vietnam.

In **Hoofdstuk 8** stelden we samenvattend dat bloedbaaninfecties bij volwassenen in Cambodia veroorzaakt wordt door moeilijk te behandelen bacteriën, en vaak met hoge mortaliteit gepaard gaan. Verschillende maatregelen dienen genomen te worden om deze situatie te verbeteren, gaande van het beschikbaar maken en reglementeren van bepaalde breedspectrumantibiotica, het aanpassen en moderniseren van behandelingsrichtlijnen, en invoeren van handhygiëne in ziekenhuizen tot betere opleiding van gezondheidswerkers en internationale samenwerking in de strijd tegen antibioticaresistentie.

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Curriculum vitae

Erika Vlieghe (°6-10-1971) studied Medicine at the Leuven Catholic University (1989-1996) followed by a postgraduate course in Tropical Medicine at the Antwerp Institute of Tropical Medicine (1996-1997). In 1997-1998 she started her professional career as a general practitioner in a remote missionary district hospital in the Karamoja, Uganda, followed by an introduction into genitourinary medicine at the Royal London Hospital in 1999. Between 1999-2004 she completed a specialization in Internal Medicine and Infectious Diseases at Leuven University after which she started working as an Infectious Diseases Physician at the Institute of Tropical Medicine and the University Hospital of Antwerp, taking care of outpatient and inpatient patients with HIV/AIDS, travel-related pathologies, general infectious diseases and antibiotic stewardship activities.

Since 2007 she has been closely involved in a capacity strengthening project on the containment of antibiotic resistance in Cambodia through a blood culture based surveillance system. In 2012 she received a 'SOFI-A'-grant from the Institute of Tropical Medicine, Antwerp to finalize her PhD on antibiotic resistance in Cambodia and its impact on local treatment guidelines.

She teaches various infectious diseases topics at undergraduate (University of Antwerp) and postgraduate level (Institute of Tropical Medicine, Antwerp) and followed additional training in Evidence Based Medicine and on Qualitative/Mixed Methods' Research at the Institute of Tropical Medicine, Antwerp.

Erika Vlieghe is married to Mark Depauw; together they have two sons: Maarten and Wout.

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